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(11) EP 1 541 680 A1

(12)

EUROPEAN PATENT APPLICATION

published in accordance with Art. 158(3) EPC

- (43) Date of publication: 15.06.2005 Bulletin 2005/24
- (21) Application number: 03794236.4
- (22) Date of filing: 04.09.2003

- (51) Int Cl.7: C12N 15/06, C07K 16/18
- (86) International application number: PCT/JP2003/011318
- (87) International publication number: WO 2004/022739 (18.03.2004 Gazette 2004/12)
- (84) Designated Contracting States:

 AT BE BG CH CY CZ DE DK EE ES FI FR GB GR
 HU IE IT LI LU MC NL PT RO SE SI SK TR
 Designated Extension States:

 AL LT LV MK
- (30) Priority: 04.09.2002 WOPCT/JP02/08999
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(54) ANTIBODY AGAINST N-TERMINAL PEPTIDE OR C-TERMINAL PEPTIDE OF GPC3 SOLUBILIZED IN BLOOD

(57) Disclosed is an antibody against a secreted form of GPC3 capable of detecting a secreted form of glypican 3 (GPC3) in a test sample. It is possible to determine whether a subject suffers from cancer, in particular hepatoma. Also disclosed is an antibody against

GPC as well as a cell disrupting agent and an anti-cancer agent comprising the same, which can disrupt cells, in particular cancer cells.

Description

Technical Field

[0001] The present invention relates to an antibody against an N-terminal peptide or C-terminal peptide of GPC3. More specifically, the invention relates to an antibody against a GPC3 N-terminal peptide of about 40 kDa as found in the soluble form of the GPC3 core protein. Additionally, the invention also relates to an antibody against a GPC3 C-terminal peptide of about 30 kDa as found in the soluble form of the GPC3 core protein.

10 Background Art

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[0002] The presence of the glypican family is reported as a new family of heparan sulfate proteoglycan existing on cell surface. Up to now, it is reported that five types of glypican (glypican 1, glypican 2, glypican 3, glypican 4 and glypican 5) exist. The members of the family have a core protein of a uniform size (about 60 kDa) and have unique cysteine residues well conserved in common, and are bound to cell membrane via glycosylphosphatidylinositol (GPI) anchor

[0003] Glypican 3 (GPC3) is known to be deeply involved in cell division during development and the control of the pattern thereof. Additionally, it is known that the GPC3 gene is highly expressed in hepatoma cell and that the GPC3 gene is possibly used as a marker of hepatocellular carcinoma.

[0004] The present inventors previously found that an anti-GPC3 antibody had an ADCC activity and a CDC activity and was useful as the therapeutic treatment of hepatoma and filed a patent application (Japanese Patent Application 2001-189443).

[0005] However, GPC3 is a membrane-bound protein and it has not been reported that a GPC3 protein of secreted form existed. Thus, no examination has been made about the use of the GPC3 protein itself as a tumor marker in blood.

Disclosure of the Invention

[0006] The present inventors found a fact that glypican 3 (GPC3) is cleaved at an amino acid residue 358 thereof or at an amino acid residue 374 thereof or a region in the vicinity of the residues. On an assumption that the soluble form of GPC3 would be secreted in the blood of hepatoma patients, the inventors established a GPC3 sandwich ELISA system to show the existence of the secreted form of GPC3 in the culture supernatant of human hepatoma cell HepG2 highly expressing GPC3. Further, the inventors successfully assayed the secreted form of GPC3 not only in the plasma of a mouse transplanted with HepG2 but also in the serum of a human hepatoma patient. Because the expression of the GPC3 gene is observed in hepatoma at an earlier stage compared with the time involving the occurrence of AFP as a hepatoma marker, the inventors considered that the detection of GPC3 would be useful for cancer diagnosis. Additionally because it appears to be hard to detect the secreted form of GPC3 with an anti-GPC3 antibody recognizing a C-terminal peptide fragment, the secreted form of GPC3 was assumed to be dominantly present as an N-terminal peptide fragment. Thus, the inventors considered that an anti-GPC3 antibody recognizing the N terminus was preferably used for detecting the secreted form of GPC3. Accordingly, the inventors made an attempt to develop an antibody recognizing the N-terminal peptide of GPC3, and thus have achieved the invention. Further, the inventors found that an antibody against the C terminus of GPC3 had a high cytotoxic activity and considered that the use of the anti-GPC3 antibody recognizing the C terminus would be preferable for disrupting cancer cell, i.e. for therapeutically treating cancer. Then, the inventors made an attempt of developing an antibody recognizing the C-terminal peptide of GPC3, and thus have achieved the invention.

[0007] Since it is observed that GPC3 is expressed in cancer cell lines other than hepatoma cell lines, such as lung cancer, colon cancer, breast cancer, prostate cancer, pancreatic cancer, and lymphoma, GPC3 may possibly be applied to the diagnosis of cancers other than hepatoma.

[0008] Specifically, the invention relates to an antibody against an N-terminal peptide of GPC3.

[0009] Additionally, the invention relates to the antibody, where the N-terminal peptide of GPC3 is a secreted form of a peptide found in blood.

[0010] Further, the invention relates to the antibody, where the N-terminal peptide of GPC3 is a peptide comprising amino acid residues 1-374 of GPC3 or a peptide comprising amino acid residues 1-358 of GPC3.

[0011] Still further, the invention relates to the antibody, which is a monoclonal antibody.

[0012] Additionally, the invention relates to the antibody, which is immobilized to an insoluble support.

[0013] Still additionally, the invention relates to the antibody, which is labeled with a labeling material.

[0014] Still more additionally, the invention relates to an antibody against a C-terminal peptide of GPC3.

[0015] Still further, the invention relates to the antibody, where the C-terminal peptide of GPC3 is a peptide comprising amino acid residues 359-580 of GPC3 or a peptide comprising amino acid residues 375-580 of GPC3.

- [0016] Still further, the invention relates to the antibody, which is a monoclonal antibody.
- [0017] Additionally, the invention relates to the antibody, which is a chimera antibody.
- [0018] Additionally, the invention relates to the antibody, which is a cytotoxic antibody.
- [0019] Still additionally, the invention relates to a cell-disrupting agent comprising the antibody.
- [0020] Additionally, the invention relates to the cell disrupting agent, where the cell is a cancer cell.
- [0021] Further, the invention relates to an anti-cancer agent comprising the antibody.
- [0022] Additionally, the invention relates to a method for inducing cytotoxicity comprising contacting a cell with the antibody.
- [0023] Still more additionally, the invention relates to the method, where the cell is a cancer cell.
- [0024] The invention is now described in detail hereinbelow.

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- [0025] The invention provides an antibody against the secreted form of glypican 3 (GPC3), which is capable of detecting the secreted form of GPC3 in a test sample. By detecting the secreted form of GPC3 in vitro in a test sample, it can be diagnosed whether or not the test subject is afflicted with cancer, particularly hepatoma.
- [0026] Detection includes quantitative or non-quantitative detection, and includes for example a simple assay for the existence of GPC3 protein, an assay for the existence of GPC3 protein at a given amount or more, and a comparative assay for the amount of GPC3 protein with the amount in other samples (for example, control sample) as a non-quantitative assay; and an assay for the concentration of the GPC3 protein and an assay for the amount of the GPC3 protein as a quantitative assay.
- [0027] The test sample includes, but is not limited to, any samples possibly containing the GPC3 protein. A sample collected from biological bodies of mammals is preferable. Further, samples collected from humans are more preferable. Specific examples of such test sample include blood, interstitial fluid, plasma, extravascular fluid, cerebrospinal fluid, synovial fluid, pleural fluid, serum, lymphoid fluid, saliva, and urine. Preferably, the test sample is blood, serum or plasma. Additionally, samples obtained from test samples, such as a culture medium of cells collected from biological bodies are also included in the test sample in accordance with the invention.
- **[0028]** The cancer to be diagnosed using the antibody against the N-terminal peptide of GPC3 in accordance with the invention includes, but is not limited to, hepatoma, pancreatic cancer, lung cancer, colon cancer, breast cancer, prostate cancer, leukemia, and lymphoma. Preferably, the cancer is hepatoma.
- **[0029]** Because the antibody against the C-terminal peptide of GPC3 in accordance with the invention has a high cytotoxic activity, the antibody can be used for disrupting cancer cells, i.e. for therapeutically treating cancer. Cancer possibly treated clinically using the antibody includes, but is not limited to, hepatoma, pancreatic cancer, lung cancer, colon cancer, breast cancer, prostate cancer, leukemia, and lymphoma. Preferably, the cancer is hepatoma.
- 1. Preparation of the anti-GPC3 antibody against the N-terminal peptide or the anti-GPC3 antibody against the C-terminal peptide
- [0030] The amino acid sequence and nucleotide sequence of GPC3 are described in Lage, H. et al., Gene 188 (1997), 151-156 or GenBank: Z37987.
- [0031] The anti-GPC3 antibody against the N-terminal peptide or the anti-GPC3 antibody against the C-terminal peptide used in the invention should be capable of specifically binding to the N-terminal peptide of the GPC3 protein or the C-terminal peptide of the GPC3 protein, respectively. The origin or type thereof (monoclonal, polyclonal) or the shape thereof is not specifically limited. Specifically, known antibodies such as mouse antibody, rat antibody, human antibody, chimera antibody and humanized antibody can be used.
- [0032] When GPC3 is cleaved at a cleavage site, the GPC3 is cut into a peptide of about 40 kDa and a peptide of about 30 kDa, which are on the N-terminal side and the C-terminal side, respectively. The cleavage site of GPC3 is the amino acid reside 358, the amino acid residue 374 or a region in the vicinity thereof. The main cleavage site is believed to be the amino acid residue 358.
- [0033] The N-terminal peptide of GPC3 is an N-terminal peptide of GPC3 and of about 40 kDa, which is found in the soluble form of the GPC3 core protein. The N-terminal peptide is preferably a peptide of an amino acid sequence comprising from Met 1 to Lys 374, or a peptide of an amino acid sequence comprising from Met 1 to Arg 358. More preferably, the N-terminal peptide is a peptide of an amino acid sequence comprising from Met 1 to Arg 358, because the main cleavage site is predicted to be at the amino acid residue 358. In accordance with the invention, fragments of the N-terminal peptide may also be employed. In this specification, the N-terminal peptide is also referred to as N-terminal fragment or N-terminal peptide fragment.
- [0034] In other words, the antibody against the N-terminal peptide of GPC3 in accordance with the invention is an antibody recognizing an epitope existing on the N-terminal peptide of the GPC3 protein. The site of the epitope recognized is not specifically limited.
 - [0035] The C-terminal peptide of GPC3 is a C-terminal peptide of GPC3 and of about 30 kDa found in the soluble form of the GPC3 core protein. Based on the cleavage site mentioned above, the C-terminal peptide is preferably a

peptide of an amino acid sequence of from Ser 359 to His 580 or a peptide of an amino acid sequence of from Val 375 to His 580. More preferably, the C-terminal peptide is a peptide of an amino acid sequence comprising from Ser 359 to His 580, because the main cleavage site is presumed to be at the site of the amino acid residue 358. In accordance with the invention, fragments of such C-terminal peptide may also be employed. In this specification, the C-terminal peptide is also referred to C-terminal fragment or C-terminal peptide fragment.

[0036] In other words, the antibody against the C-terminal peptide of GPC3 in accordance with the invention is an antibody recognizing an epitope existing on the C-terminal peptide of the GPC3 protein, and the site of the epitope recognized is not limited.

[0037] The antibody may be a polyclonal antibody but is preferably a monoclonal antibody.

[0038] The anti-GPC3 N-terminal peptide antibody or the anti-GPC3 C-terminal peptide antibody for use in accordance with the invention can be obtained as a polyclonal antibody or a monoclonal antibody, using known techniques. The anti-GPC3 antibody for use in accordance with the invention is preferably a monoclonal antibody derived from mammals. The monoclonal antibody derived from mammals includes those produced by hybridoma, and those generated in hosts transformed with expression vectors carrying the antibody gene by genetic engineering technology.

[0039] Hybridoma producing a monoclonal antibody is prepared essentially using known techniques as follows. An animal is immunized by a conventional immunization method using GPC3 as a sensitizing antigen to obtain an immune cell, which is then fused to a known parent cell by a conventional cell fusion method. Fused cells are screened for monoclonal antibody-generating cells by a conventional screening method.

[0040] Specifically, a monoclonal antibody is prepared as follows.

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[0041] First, GPC3 for use as a sensitizing antigen for obtaining antibody is prepared by expressing the GPC3 (MXR7) gene/amino acid sequence disclosed in Lage, H. et al., Gene 188 (1997), 151-156. Particularly, the gene sequence encoding GPC3 is inserted in a known expression vector to transform an appropriate host cell, then the intended human GPC3 protein is purified from the host cell or a culture supernatant thereof.

[0042] Additionally, naturally occurring GPC3 may also be purified and used.

[0043] Then, the purified GPC3 protein is used as a sensitizing antigen. The whole GPC3 protein may be used as a sensitizing antigen. Because an antibody against the N-terminal peptide of the GPC3 protein and an antibody against the C-terminal peptide thereof are also induced in this case, the antibody against the N-terminal peptide of the GPC3 protein and the antibody against the C-terminal peptide thereof may be separately selected. Alternatively, a partial N-terminal peptide of GPC3 or a partial C-terminal peptide thereof may also be used as a sensitizing antigen. In that case, such partial peptide may be obtained by chemical synthesis on the basis of the amino acid sequence of human GPC3 or by inserting a part of the GPC3 gene into an expression vector or by degrading naturally occurring GPC3 with proteases. The part of GPC3 for use as a partial peptide is the N-terminal GPC3 peptide. A smaller peptide fragment containing the epitope in the part may also be used. Further, a C-terminal peptide of GPC3 may be used as a partial peptide, and a smaller peptide fragment containing the epitope in the part may also be used.

[0044] Mammals for immunization with a sensitizing antigen are preferably selected, with taking account of the compatibility with parent cells for use in cell fusion. The mammals used for immunization preferably include, but are not limited to, rodents such as mouse, rat, hamster or rabbit or monkey.

[0045] For immunization of animals with a sensitizing antigen, known methods may be employed. Generally, for example, a sensitizing antigen is injected intraperitoneally or subcutaneously in mammals. Specifically, a sensitizing antigen is diluted with or suspended in PBS (phosphate-buffered saline) or physiological saline or the like, to an appropriate volume, and mixed with an appropriate volume of conventional adjuvants, such as Freund's complete adjuvant. After emulsification, the emulsified mixture is administered to mammals several times every 4 to 21 days. Additionally, an appropriate carrier may be used during the immunization with a sensitizing antigen. In case that a partial peptide of a very small molecular weight is to be used as a sensitizing antigen, the partial peptide may preferably be bound to carrier proteins, such as albumin and keyhole limpet hemocyanin upon immunization.

[0046] After mammals are immunized as above and the increase in the level of a desired antigen in serum is observed, immune cells are collected from the mammals, which are then subjected to cell fusion. Preferably, the immune cell is splenocyte.

[0047] As another parent cell to be fused to the immune cell, mammalian myeloma cell may be used. As the myeloma cell, known various cell lines are preferably used, including for example P3 (P3x63Ag8. 653) (J. Immunol. (1979) 123, 1548-1550), P3x63Ag8U. 1 (Current Topics in Microbiology and Immunology (1978) 81, 1-7), NS-1 (KohlerG. andMilstein, C. Eur. J. Immunol. (1976) 6, 511-519), MPC-11 (Margulies, D. H. et al., Cell (1976) 8, 405-415), SP2/0 (Shulman, M. et al., Nature (1978) 276, 269-270), F0 (de St. Groth, S. F. et al., J. Immunol. Methods (1980) 35, 1-21), S194 (Trowbridge, I. S. J. Exp. Med. (1978) 148, 313-323), and R210 (Galfre, G. et al., Nature (1979) 277, 131-133).

[0048] The cell fusion of the immune cell to the myeloma cell is essentially done by known methods, for example the method of Kohler & Milstein et al. (Kohler G. and Milstein C., Methods Enzymol. (1981) 73, 3-46).

[0049] More specifically, the cell fusion is carried out in conventional nutritious culture media in the presence of a cell fusion stimulator. Cell fusion stimulator includes, for example, polyethylene glycol (PEG) and Sendai virus (HVJ).

If desired, auxiliary agents such as dimethylsulfoxide can be added and used so as to enhance the fusion efficiency. **[0050]** The ratio of an immune cell and a myeloma cell to be used can appropriately be determined. For example, an immune cell at a ratio of 1- to 10-fold a myeloma cell is preferable. Culture medium for use in the cell fusion includes, for example, RPMI1640 and MEM, and other conventional culture media suitable for the growth of myeloma cell lines. Further, auxiliary serum agents such as fetal calf serum (FCS) may be used in combination.

[0051] The cell fusion can be done by thoroughly mixing predetermined amounts of immune cells and myeloma cells in the culture medium, adding the resulting mixture to a PEG solution (for example, mean molecular weight of about 1,000 to 6,000) preliminarily heated to about 37 °C, generally to a concentration of 30 to 60 w/v %, and subsequently mixing the mixture to allow the intended fusion cell (hybridoma) to be formed. Subsequently, a cell fusion agent and the like unpreferable for the growth of hybridoma are removed by adding appropriate culture medium sequentially and centrifuging the mixture to discard the supernatant, and repeating the procedures described above.

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[0052] The hybridoma thus obtained is selected by culturing in a conventional selective culture medium, such as HAT medium (containing hypoxanthine, aminopterin and thymidine). The culturing in the HAT medium is continued for a sufficient period of time (typically several days to several weeks) for killing cells (non-fused cells) other than the intended hybridoma cell. Then, a conventional limited dilution method is carried out for screening and single cloning of a hybridoma producing the intended antibody.

[0053] The screening and the single cloning of the hybridoma may be done by a screening method on the basis of known antigen-antibody reactions. The antigen is bound to carriers such as beads made of polystyrene and the like, or commercially available 96-well microtiter plates, and reacted with a culture supernatant of the hybridoma. After rinsing the carriers, an enzyme-labeled secondary antibody is added to the plate to determine whether an intended antibody reacting with the sensitizing antigen is contained in the culture supernatant. The hybridoma producing the intended antibody can be cloned by limited dilution method. The N-terminal peptide of GPC3 or a fragment thereof or the C-terminal peptide of GPC3 or a fragment thereof may be used as the antigen for screening.

[0054] In addition to obtaining hybridoma by immunizing an animal except humans with an antigen, a human antibody may be prepared by another method. Human lymphocyte is sensitized with GPC3 in vitro and is then fused to myeloma cell with a permanent division potency derived from humans, to obtain a desired human antibody with a binding activity to the N-terminal peptide of GPC3 or the C-terminal peptide of GPC3 (see JP-B-1-59878). Further, a human antibody against the N-terminal peptide of GPC3 or the C-terminal peptide of GPC3 may be obtained by administering GPC3 as an antigen to a transgenic animal bearing all the repertories of the genes of human antibodies to obtain a cell producing an anti-GPC3 antibody against the N-terminal peptide or a cell producing an anti-GPC3 antibody against the C-terminal peptide, and then immortalizing the cell (see International Publications WO 94/25585, WO 93/12227, WO 92/03918, and WO 94/02602).

[0055] The hybridoma producing the monoclonal antibody thus prepared can be subcultured in a conventional culture medium and can be stored in liquid nitrogen for a long period of time.

[0056] One method for obtaining the monoclonal antibody from the hybridoma involves culturing the hybridoma by a conventional method and obtaining the monoclonal antibody from a culture supernatant thereof. Another method involves administering the hybridoma to an animal compatible to the hybridoma for proliferation and obtaining the monoclonal antibody in the form of ascites. The former method is suitable for obtaining the antibody at high purity, while the latter method is suitable for large-scale production of the antibody.

[0057] In accordance with the invention, a monoclonal antibody includes a recombinant antibody produced by gene recombinant technology. A recombinant antibody can be generated by cloning the gene of the antibody from the hybridoma, integrating the gene into an appropriate vector, introducing the gene into a host, and allowing the recombinant antibody to be produced by the host (see for example Vandamme, A. M. et al., Eur. J. Biochem. (1990) 192, 767-775, 1990). Specifically, mRNA encoding the variable (V) region of the anti-GPC3 N-terminal peptide or the anti-GPC3 C-terminal peptide is isolated from the hybridoma generating the anti-GPC3 N-terminal peptide antibody or the hybridoma generating the anti-GPC3 C-terminal peptide antibody, respectively. mRNA isolation can be done by known methods. For example, total RNA is prepared by guanidine ultra-centrifugation method (Chirgwin, J. M. et al., Biochemistry (1979) 18, 5294-5299) or AGPC method (Chomczynski, P. et al., Anal. Biochem. (1987) 162, 156-159), from which the intended mRNA is prepared using the mRNA purification kit (manufactured by Pharmacia). Alternatively, mRNAcan directly be prepared using QuickPrep mRNA purification kit (manufactured by Pharmacia).

[0058] cDNA of the V region of the antibody is synthesized from the resulting mRNA, using reverse transcriptase. cDNA can be synthesized, using AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (manufactured by Seikagaku Corporation). cDNA can also be synthesized and amplified using 5'-AmpliFinder Race Kit (manufactured by Clontech) and 5'-RACE method using PCR (Frohman, M.A. et al., Proc. Natl. Acad. Sci. USA (1988) 85, 8998-9002; Belyavsky, A. et al., Nucleic Acids Res. (1989) 17, 2919-2932).

[0059] The intended DNA fragment is purified from the resulting PCR product and linked to vector DNA. A recombinant vector is prepared from the vector DNA and introduced in Escherichia coli and the like to select a colony for preparation of a desired recombinant vector. Subsequently, the nucleotide sequence of the intended DNA can be

confirmed by known methods, for example dideoxynucleotide chain termination method.

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[0060] After DNA encoding the V region of the intended anti-GPC3 N-terminal peptide antibody or the intended anti-GPC3 C-terminal peptide antibody is obtained, the DNA is inserted into an expression vector containing DNA encoding the desired constant region (C region) of the antibody.

[0061] So as to produce the anti-GPC3 N-terminal peptide antibody or the anti-GPC3 C-terminal peptide antibody for use in accordance with the invention, the gene of the antibody is introduced into an expression vector such that the gene is expressed under the control of an expression-regulating region, for example enhancer and promoter. Then, a host cell is transformed with the expression vector, to express the antibody.

[0062] The gene of the antibody may be expressed by separately inserting DNA encoding the heavy chain (H chain) of the antibody and DNA encoding the light chain (L chain) thereof in expression vectors to simultaneously transform a host cell, or by inserting DNAs encoding the H chain and the L chain in a single expression vector to transform a host cell (see WO 94/11523).

[0063] Additionally, not only suchhost cells but also transgenic animal can be used for generating a recombinant antibody. For example, the gene of the antibody is inserted intermediately into a gene encoding a protein (e.g., goat β casein) generated inherently in milk to prepare a fusion gene. The DNA fragment comprising the fusion gene with the gene of the antibody as inserted therein is injected in a goat embryo, which is introduced in a female goat. The desired antibody is obtained from the milk produced by a transgenic goat born from the goat having received the embryo or a progeny thereof. So as to increase the amount of milk containing the desired antibody as produced by the transgenic goat, hormone may appropriately be administered to the transgenic goat (Ebert, K. M. et al., Bio/Technology (1994) 12, 699-702)

[0064] In accordance with the invention, artificially modified recombinant antibodies, for example a chimera antibody (e.g., humanized antibody) may also be used. These modified antibodies can be produced, using existing methods. In case that the antibody of the invention is to be used as an antibody for therapeutic treatment, the genetic recombinant type antibody is preferably used.

[0065] Chimera antibody can be obtained by linking the DNA encoding the V region of the antibody as obtained in the manner described above to DNA encoding the C region of a human antibody, inserting the resulting DNA in an expression vector, and introducing the vector in a host for production of the antibody. Using this existing method, a chimera antibody useful in accordance with the invention can be obtained.

[0066] Humanized antibody is also referred to as reshaped human antibody and is prepared by transplanting the complementarity determining region (CDR) of an antibody of mammals except humans, for example mouse, into the complementarity determining region of a human antibody. General genetic recombination techniques thereof are also known in the art (see European Patent Application EP 125023; WO 96/02576).

[0067] Specifically, a DNA sequence designed such that the CDR of mouse antibody can be linked to the framework region (FR) of human antibody is synthetically prepared by PCR, using several oligonucleotides prepared in such a manner that the oligonucleotides might have parts overlapped with the terminal regions of both CDR and FR (see the method described in WO 98/13388).

[0068] The FR region of human antibody to be liked to CDR is selected such that the CDR can form a good antigen binding site. If necessary, the amino acids in the FR in the V region of the antibody may be substituted, so that the CDR of the reshaped human antibody may form an appropriate antigen binding site (Sato, K. et al., Cancer Res. (1993) 53, 851-856).

[0069] As the C regions of chimera antibody and humanized antibody, those of human antibody are used; for example, C γ 1, C γ 2, C γ 3, and C γ 4 can be used for the H chain, while C κ and C λ can be used for the L chain. So as to improve the stability of the antibody or the production thereof, the C region of human antibody may be modified.

[0070] Preferably, the chimera antibody contains a sequence of an antibody derived from mammals except humans in the V region, and contains a sequence derived from a human antibody in the C region.

[0071] Humanized antibody comprises the CDR of an antibody derived from mammals except humans, and the FR and C regions derived from a human antibody. Because the antigenicity of chimera antibody such as humanized antibody is reduced in humans, chimera antibody is useful as an active component of a therapeutic agent of the invention.

[0072] The antibody for use in accordance with the invention is not only the whole antibody molecule but also a fragment of the antibody or a modified product thereof, including divalent antibody and monovalent antibody, as long as such fragment or such modified product can bind to the GPC3 N-terminal peptide or the GPC3 C-terminal peptide. For example, the antibody fragment includes Fab, F(ab')2, Fv, Fab/C having one Fab and complete FC, or single chain Fv (scFv) where Fv of the H chain and the L chain are linked via an appropriate linker. Specifically, the antibody is treated with enzymes, for example papain and pepsin, to generate antibody fragments. Otherwise, genes encoding these antibody fragments are constructed, introduced in an expression vector and expressed in an appropriate host cell (see for example, Co, M. S. et al., J. Immunol. (1994) 152, 2968-2976; Better, M. & Horwitz, A. H. Methods in Enzymology (1989) 178, 476-496, Academic Press, Inc.; Plueckthun, A. & Skerra, A. Methods in Enzymology (1989) 178, 476-496, Academic Press, Inc.; Lamoyi, E., Methods in Enzymology (1989) 121, 652-663; Rousseaux, J. et al.,

Methods in Enzymology (1989) 121, 663-669; Bird, R. E. et al., TIBTECH (1991) 9, 132-137).

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[0073] ScFv can be obtained by linking the V region of the H chain and the V region of the L chain of an antibody. In this scFv, the V region of the H chain and the V region of the L chain are linked together via a linker, preferably a peptide linker (Huston, J. S. et al., Proc. Natl. Acad. Sci. U.S.A. (1988) 85, 5879-5883). The V region of the H chain and the V region of the L chain in scFv may be derived from any antibodies described herein. Any appropriate single-stranded peptide comprising 12 to 19 amino acid residues may be used as the peptide linker for linking the V regions. [0074] DNA encoding scFv is obtained by first amplifying DNA encoding the H chain or the V region of the H chain and the DNA encoding the L chain or the V region of the L chain by using as a template a portion of DNA encoding all the sequences thereof or a desired amino acid sequence therein and a pair of primers defining both the ends, and then amplifying the DNA with DNA encoding the peptide linker and a pair of primers defined in such a manner that both the ends of the peptide linker may be linked respectively to the H chain and the L chain.

[0075] Once the DNA encoding scFv is prepared, an expression vector carrying the DNA and a host transformed with the expression vector can be obtained by conventional methods. scFv can be obtained using the host by conventional methods.

[0076] The antibody fragments can be generated by obtaining and expressing the gene in the same manner as described above and allowing a host to produce the fragments. The "antibody" in accordance with the invention includes such antibody fragments.

[0077] There may also be used a modified product of the antibody, for example, anti-glypican antibodies conjugated with various molecules such as labeling substances, toxin, and radioactive materials. The "antibody" in accordance with the invention includes these modified antibodies. Such modified antibodies can be obtained by chemical modification of an antibody. Methods for modifying antibodies have already been established in the art.

[0078] Further, the antibody for use in accordance with the invention may be a bispecific antibody. The bispecific antibody may include those having antigen binding sites recognizing different epitopes on the N-terminal peptide of GPC3 or the C-terminal peptide of GPC3. Alternatively, one of the antigen binding sites recognizes the N-terminal peptide of GPC3 or the C-terminal peptide of GPC3, while the other antigen binding site may recognize a labeling substance and the like. Such bispecific antibody can be prepared or obtained by linking HL pairs of two types of antibodies or by fusing hybridomas generating different monoclonal antibodies together to prepare a fusion cell capable of producing a bispecific antibody. Further, such bispecific antibody can be prepared by genetic engineering technique.

[0079] In accordance with the invention, an antibody with a modified sugar chain may also be used for the purpose of enhancing cytotoxic activity. Modification technique of the sugar chain of antibody is known in the art(for example, WO 00/61739, WO 02/31140, etc.).

[0080] The antibody gene constructed in the manner described above can be expressed and obtained by known methods. In case of a mammalian cell, a conventional useful promoter, the antibody gene to be expressed and poly (A) signal downstream the 3' side thereof are functionally linked for the expression. For example, the promoter/enhancer includes human cytomegalovirus immediate early promoter/enhancer.

[0081] Additionally, the promoter/enhancer for use in the expression of the antibody for use in accordance with the invention includes, for example, virus promoters including retrovirus, polyoma virus, adenovirus and simian virus 40 (SV40)/enhancer or promoters derived from mammalian cells such as human elongation factor la (HEFla)/enhancer. [0082] In case of using SV40 promoter/enhancer, gene expression can readily be done by the method of Mulligan et al. (Nature (1979) 277, 108). In case of using the HEFla promoter/enhancer, gene expression can readily be done by the method of Mizushima et al. (Nucleic Acids Res. (1990) 18, 5322).

[0083] In case of Escherichia coli, a useful conventional promoter, a signal sequence for antibody secretion and an antibody gene to be expressed are functionally linked for expressing the gene. The promoter includes for example lacz promoter and araB promoter. In case that lacz promoter is to be used, the gene can be expressed by the method of Ward et al. (Nature (1098), 341, 544-546; FASEB J. (1992) 6, 2422-2427). In case that araB promoter is to be used, the gene can be expressed by the method of Better et al. (Science (1988) 240, 1041-1043).

[0084] As the signal sequence for antibody secretion, pelB signal sequence (Lei, S. P. et al. J. Bacteriol. (1987) 169, 4379) may be used when the antibody is generated in the periplasm of Escherichia coli. After the antibody generated in the periplasm is separated, the structure of the antibody is appropriately refolded for use.

[0085] As the replication origin, those from SV40, polyoma virus, adenovirus and bovine papilloma virus (BPV) may be used. For amplification of the copy number of the gene in a host cell system, the expression vector may carry a selective marker, for example, aminoglycoside transferase (APH) gene, thymidine kinase (TK) gene, Escherichia coli xanthine guanine phosphoribosyl transferase (Ecogpt) gene and dehydrofolate reductase (dhfr) gene.

[0086] So as to produce the antibody for use in accordance with the invention, an appropriate expression system, for example eukaryotic cell or prokaryotic cell system can be used. The eukaryotic cell includes for example established animal cell lines such as mammalian cell lines, insect cell lines, fungal cells and yeast cells. The prokaryotic cell includes for example bacterial cells such as Escherichia coli cell.

[0087] Preferably, the antibody for use in accordance with the invention is expressed in mammalian cells, for example

CHO, COS, myeloma, BHK, Vero, and HeLa cell.

[0088] The transformed host cell is cultured in vitro or in vivo to produce the intended antibody. The host cell may be cultured by known methods. As the culture medium, for example, DMEM, MEM, RPMI 1640 and IMDM can be used. Auxiliary serum fluid such as fetal calf serum (FCS) may also be used in combination.

[0089] The antibody expressed and generated as described above can be separated from such cells or host animals and can then be purified to homogeneity. The antibody for use in accordance with the invention can be separated and purified using an affinity column. A protein A column includes, for example, Hyper D, POROS, Sepharose F. F. (manufactured by Pharmacia). Additionally, any separation and purification methods generally used for protein may be employed in the invention. For example, chromatography columns other than affinity column, filter, ultrafiltration, saltingout, and dialysis may be used in combination to separate and purify the antibody (Antibodies A Laboratory Manual, Ed. Harlow, David Lane, Cold Spring Harbor Laboratory, 1988).

2. Detection of GPC3

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[0090] Using the antibody against the N-terminal peptide of GPC3 in accordance with the invention, GPC3 in a test sample can be detected.

[0091] GPC3 to be detected using the antibody of the invention includes, but is not limited to, full-length GPC3 and fragments thereof. So as to detect GPC3 fragments, preferably, a fragment of the N-terminal peptide is detected.

[0092] The method for detecting the GPC3 protein in a test sample is not specifically limited. The GPC3 protein is preferably detected by an immunoassay method using the anti-GPC3 N-terminal peptide antibody. The immunoassaymethod includes, for example, radioimmunoassay, enzyme immunoassay, fluorescent immunoassay, luminescent immunoassay, immunoprecipitation method, immunonephelometry, western blot technique, immunostaining, and immunodiffusion method. Preferably, the immunoassay method is enzyme immunoassay. Particularly preferably, the immunoassay method is enzyme-linked immunosorbent assay (ELISA) (for example, sandwich ELISA). The immunoassay method such as ELISA as described above can be done by a person skilled in the art according to known methods

[0093] General detection methods using the anti-GPC3 N-terminal peptide antibody to detect the GPC3 protein in a test sample involve, for example, immobilizing the anti-GPC3 N-terminal peptide antibody on a support, adding a test sample to the support for incubation to bind the GPC3 protein to the anti-GPC3 N-terminal peptide antibody, rinsing the support and detecting the GPC3 protein bound through the anti-GPC3 N-terminal peptide antibody to the support.

[0094] The support for use in accordance with the invention includes, for example, insoluble polysaccharides such as agarose and cellulose, synthetic resins such as silicone resin, polystyrene resin, polyacrylamide resin, nylon resin and polycarbonate resin, and insoluble supports such as glass. These supports can be used in the forms of beads and plates. In case of beads, a column packed with beads can be used. In case of plates, multi-well plate (for example, 96-well multi-well plate) and biosensor chip can be used. The anti-GPC3 N-terminal peptide antibody can be bound to the support by general methods such as chemical binding and physical adsorption. Such supports are commercially available.

[0095] The binding of the anti-GPC3 N-terminal peptide antibody to the GPC3 protein is generally done in buffers. For example, phosphate buffer, Tris buffer, citric acid buffer, borate salt buffer, and carbonate salt buffer may be used as a buffer. Incubation may be carried out under conditions commonly used, for example, 4 °C to ambient temperature for one hour to 24 hours. Rinsing after incubation may be done using any solutions which do not inhibit the binding of the GPC3 protein to the anti-GPC3 N-terminal peptide antibody. For example, buffers containing surfactants such as Tween 20 may be used.

[0096] For the method for detecting the GPC3 protein in accordance with the invention, a control sample may be placed in addition to a test sample containing GPC3 protein to be detected. The control sample includes, for example, a negative control sample containing no GPC3 protein or a positive control sample containing the GPC3 protein. In this case, the GPC3 protein in the test sample can be detected by comparison with the results obtained using the negative control sample containing no GPC3 protein and the results obtained using the positive control sample containing the GPC3 protein. Additionally, a series of control samples having serially varied concentrations are prepared and the results of detection in the individual control samples are obtained in numerical figure to prepare a standard curve. Based on the standard curve, the GPC3 protein contained in the test sample can be determined quantitatively, based on the numerical figure about the test sample.

[0097] A preferable embodiment of the detection of the GPC3 protein bound through the anti-GPC3 N-terminal peptide antibody to the support includes a method using the anti-GPC3 N-terminal peptide antibody labeled with a labeling substance.

[0098] For example, a test sample is put in contact with the anti-GPC3 antibody immobilized on a support, which is then rinsed, to detect the GPC3 protein using a labeled antibody specifically recognizing the GPC3 protein.

[0099] In this case, the anti-GPC3 N-terminal peptide antibody immobilized on the support and anti-GPC3 N-terminal

peptide C antibody labeled with a labeling substance may recognize the same epitope of the GPC3 molecule, but preferably recognize different epitopes.

[0100] The anti-GPC3 N-terminal peptide antibody can be labeled by generally known methods. Any labeling substances known to a person skilled in the art can be used, including for example fluorescent dye, enzyme, coenzyme, chemiluminescent substance and radioactive substance. Specific examples thereof include for example radioisotopes (32 P, 14 C, 125 I, 3 H and 131 I), fluorescein, rhodamine, dansylchloride, umbelliferone, luciferase, peroxidase, alkaline phosphatase, β -galactosidase, β -glucosidase, horse radish peroxidase, glucoamylase, lysozyme, saccharide oxidase, microperoxidase, and biotin. Preferably, in the case that biotin is used as a labeling substance, avidin bound with enzymes such as alkaline phosphatase is further added after the addition of a biotin-labeled antibody. For binding the anti-GPC3 antibody with a labeling substance, any of the known methods such as glutaraldehyde method, maleimide method, pyridyl disulfide method and periodate method may be used.

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[0101] Specifically, a solution containing the anti-GPC3 N-terminal peptide antibody is added to a support, such as a plate, to immobilize anti-GPC3 N-terminal peptide antibody. After rinsing the plate, the plate is blocked with for example BSA, so as to prevent non-specific protein binding. After rinsing again, a test sample is added to the plate. After incubation, the plate is rinsed, to which the labeled anti-GPC3 antibody is added. After appropriate incubation, the plate is rinsed and the labeled anti-GPC3 antibody remaining on the plate is detected. The detection can be done by methods known to a person skilled in the art. For example, in case of labeling with a radioactive substance, the detection can be done by a liquid scintillation or a RIA method. In case of labeling with an enzyme, a substrate for the respective enzyme is added to detect enzymatic substrate changes via for example color development by spectrophotometer. Specific examples of such substrate include 2,2-adinobis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS), 1,2-phenylenediamine (ortho-phenylenediamine), and 3,3',5,5'-tetramethylbenzidine (TME). In case of labeling with a fluorescent substance, the fluorescent substance can be detected with fluorophotometer.

[0102] A particularly preferable embodiment of the method for detecting the GPC3 protein in accordance with the invention involves using anti-GPC3 N-terminal peptide antibody labeled with biotin and avidin. Specifically, a solution containing anti-GPC3 N-terminal peptide antibody is added to a support such as plate, to immobilize the anti-GPC3 N-terminal peptide antibody. After rinsing the plate, the antibody is blocked with for example BSA to prevent non-specific protein binding. After rinsing again, a test sample is added to the plate. After incubation, the plate is rinsed, and the biotin-labeled anti-GPC3 antibody is added. After appropriate incubation, the plate is rinsed, and avidin conjugated to an enzyme, such as alkaline phosphatase or peroxidase is added. After incubation, the plate is rinsed, a substrate corresponding to each enzyme conjugated to avidin is added, and the GPC3 protein is detected using an enzymatic substrate change as an indicator.

[0103] Another embodiment of the method for detecting the GPC3 protein in accordance with the invention involves using a primary antibody specifically recognizing the GPC3 protein and a secondary antibody specifically recognizing the primary antibody.

[0104] For example, a test sample is put in contact with the anti-GPC3 N-terminal peptide antibody immobilized on a support. After incubation, the support is rinsed and the GPC3 protein bound to the support after rinsing is detected using a primary anti-GPC3 antibody and a secondary antibody specifically recognizing the primary antibody. In this case, the secondary antibody is preferably labeled with a labeling substance.

[0105] Specifically, a solution containing anti-GPC3 N-terminal peptide antibody is added to a support, such as plate, to immobilize the anti-GPC3 N-terminal peptide antibody. After rinsing the plate, the antibody is blocked with for example BSA to prevent non-specific protein binding. After rinsing again, a test sample is added to the plate. After incubation, the plate is rinsed and a primary anti-GPC3 antibody is added. After appropriate incubation, the plate is rinsed and a secondary antibody specifically recognizing the primary antibody is added. After appropriate incubation, the plate is rinsed and the secondary antibody remaining on the plate is detected. The detection of the secondary antibody can be done by the methods described above.

[0106] Still another embodiment of the method for detecting the GPC3 protein in accordance with the invention involves using an aggregation reaction. In this method, GPC3 can be detected using a carrier sensitized with the anti-GPC3 N-terminal peptide antibody. Any carriers may be used as the carrier to be sensitized with the antibody, as far as the carrier is insoluble and stable and does not undergo non-specific reaction. For example, latex particle, bentonite, collodion, kaolin and immobilized sheep erythrocyte may be used. Latex particle is preferably used. Latex particles include, for example, polystyrene latex particle, styrene-butadiene copolymer latex particle, and polyvinyltoluene latex particle. Polystyrene latex particle is preferably used. After the sensitized particle is mixed with a sample and agitated for a given period of time, GPC3 can be detected by observing the aggregation under naked eyes since the aggregation level of such particle is higher as the GPC3 antibody is contained at a higher concentration in the sample. Additionally, the turbidity due to the aggregation can be measured with spectrophotometer and the like, to detect GPC3.

[0107] Another embodiment of the method for detecting the GPC3 protein in accordance with the invention involves using a biosensor utilizing surface plasmon resonance phenomenon enables the observation of the protein-protein interaction as surface plasmon resonance signal

on real time using a trace amount of protein without labeling. For example, the binding of the GPC3 protein to the anti-GPC3 N-terminal peptide antibody can be detected by using biosensors such as BIAcore (manufactured by Pharmacia). Specifically, a test sample is put in contact with a sensor chip having the anti-GPC3 N-terminal peptide antibody immobilized thereon, and the GPC3 protein bound to the anti-GPC3 N-terminal peptide antibody is detected as the change of the resonance signal.

[0108] The detection methods in accordance with the invention may be automated using various automatic laboratory apparatuses, so that a large volume of samples can be tested at a time.

[0109] It is an objective of the invention to provide a diagnostic reagent or kit for detecting GPC3 protein in a test sample for cancer diagnosis. The diagnostic reagent or kit contains at least the anti-GPC3 N-terminal peptide antibody. In case that the diagnostic reagent or kit is based on EIA, a carrier for immobilizing the antibody may be contained, or the antibody may be preliminarily bound to a carrier. In case that the diagnostic reagent or kit is based on the aggregation method using carriers such as latex, the reagent of kit may contain a carrier having the antibody adsorbed thereon. Additionally, the kit may appropriately contain, for example, a blocking solution, a reaction solution, a reaction-terminating solution and reagents for treating sample.

- 3. Disruption of cancer cell using the anti-GPC3 C-terminal peptide antibody and cancer therapy using the same
- (1) Determination of antibody activity
- 20 [0110] The antigen binding activity of the antibody for use in accordance with the invention may be assayed using known techniques (Antibodies A Laboratory Manual. Ed. Harlow, David Lane, Cold Spring Harbor Laboratory, 1988) and an activity of inhibiting the ligand-receptor binding thereof (Harada, A. et al., International Immunology (1993) 5, 681-690).
 - [0111] A method for assaying the antigen binding activity of the anti-GPC3 C-terminal peptide antibody for use in accordance with the invention includs ELISA (enzyme-linked immunosorbent assay), EIA (enzyme immunoassay), RIA (radioimmunoassay) and fluorescent antibody method. In enzyme immunoassay, a sample containing the anti-GPC3 C-terminal peptide antibody, for example a culture supernatant of a cell producing the anti-GPC3 C-terminal peptide antibody or the purified antibody is added to a plate coated with the GPC3 C-terminal peptide. A secondary antibody labeled with an enzyme such as alkali phosphatase is added and the plate is incubated and rinsed, then an enzyme substrate such as p-nitrophenylphosphoric acid is added to measure the absorbance and assess the antigen binding activity.
 - [0112] So as to determine the activity of the antibody for use in accordance with the invention, the neutralization activity of the anti-GPC3 C-terminal peptide antibody is measured.
- (2) Cytotoxicity

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- [0113] For therapeutic purpose, the antibody for use in accordance with the invention preferably has the ADCC activity or the CDC activity as cytotoxicity.
- [0114] The ADCC activity can be assayed by mixing an effector cell, a target cell and the anti-GPC3 C-terminal peptide antibody together and examining the ADCC level. As the effector cell, cell such as mouse splenocyte and mononuclear cell separated from human peripheral blood or bone marrow can be utilized. As the target cell, a human cell line such as human hepatoma line HuH-7 can be used. The target cells are preliminarily labeled with ⁵¹Cr and incubated with the anti-GPC3 C-terminal peptide antibody, then effector cells at an appropriate ratio is added to the target cells and incubated. After incubation, the supernatant is collected to count the radioactivity in the supernatant, to assay the ADCC activity.
 - **[0115]** Further, the CDC activity can be assayed by mixing the labeled target cell described above with the anti-GPC3 C-terminal peptide antibody, subsequently adding complement, and counting the radioactivity in the supernatant after incubation.
- [0116] The Fc moiety is needed for the antibody to exert the cytotoxicity. In case that the inhibitor of cell proliferation in accordance with the invention utilizes the cytotoxicity of the antibody, thus, the anti-GPC3 C-terminal peptide antibody for use in accordance with the invention preferably contains the Fc moiety.
 - (3) Cell disruption
- [0117] The anti-GPC3 C-terminal peptide antibody of the invention may also be used for cell disruption, particularly the disruption of cancer cell. Further, the anti-GPC3 C-terminal peptide antibody of the invention can be used as an anticancer agent. Cancers to be therapeutically treated and prevented by the antibody of the invention include, but are not limited to, hepatoma, lung cancer, colon cancer, breast cancer, prostate cancer, pancreatic cancer and lymphoma,

preferably Hepatoma.

- (4) Administration method and pharmaceutical formulation
- [0118] The cell disrupting agent or anticancer agent in accordance with the invention is used for the purpose of therapeutically treating or ameliorating diseases caused by abnormal cell growth, particularly cancer.
 - **[0119]** The effective dose is selected within a range of 0.001 mg to 1,000 mg per 1 kg body weight. Also the effective dose is selected within a range of 0.01 mg to 100,000 mg/body weight per patient. However, the dose of the therapeutic agents containing the anti-GPC3 C-terminal peptide antibody of the invention are not limited to the above doses.
- [0120] The timing for administering the therapeutic agent of the invention is either before or after the onset of clinical symptoms of the diseases.
 - **[0121]** The therapeutic agent comprising the anti-GPC3 C-terminal-peptide antibody in accordance with the invention as an active component can be formulated by a conventional method (Remington's Pharmaceutical Science, latest edition, Mark Publishing Company, Easton, USA), and may also contain pharmaceutically acceptable carriers and additives.
 - [0122] Examples of such carriers and pharmaceutical additives include water, pharmaceutically acceptable organic solvents, collagen, polyvinyl alcohol, polyvinyl pyrrolidone, carboxyvinyl polymer, carboxymethyl cellulose sodium, polyacrylate sodium, sodium alginate, water-soluble dextran, carboxymethyl starch sodium, pectin, methyl cellulose, ethyl cellulose, gum xanthan, gum arabic, casein, agar, polyethylene glycol, diglycerin, glycerin, propylene glycol, vaseline, paraffin, stearyl alcohol, stearic acid, human serum albumin (HSA), mannitol, sorbitol, lactose and surfactants acceptable as pharmaceutical additives.
 - [0123] In practice, an additive or a combination thereof is selected depending on the dosage form of the therapeutic agent of the invention. However, the additive is not limited to those described above. In case that the therapeutic agent is to be used in an injection formulation, the purified anti-GPC3 C-terminal peptide antibody of the invention is dissolved in a solvent, such as physiological saline, buffers, and glucose solution, and adsorption preventing agents such as Tween 80, Tween 20, gelatin and human serum albumin is added. Alternatively, the therapeutic agent is provided in a freeze-dried form as a dosage form to be dissolved and reconstituted prior to use. As excipients for freeze-drying, for example, sugar alcohols such as mannitol and glucose and sugars may be used.
- 30 Brief Description of the Drawings

[0124]

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- Fig. 1 shows bar graphs depicting the results of the analysis of GPC3 mRNA expression using Gene Chip, where Fig. 1A depicts GPC3 expression and Fig. 1B depicts the expression of alpha-fetoprotein (AFP). NL, CH, LC, WD, MD and PD on the holizontal axis represent normal liver, inflammatory lesion of hepatitis, lesion of liver cirrhosis, well-differentiated cancer, moderately differentiated cancer and poorly differentiated cancer, respectively.
 - Fig. 2 shows images of purified soluble GPC3 of heparan sulfate adduct type and the GPC3 core protein, as stained with CBB.
- 40 Fig. 3 shows bar graphs depicting the expression of the GPC3 gene in human hepatoma.
 - Fig. 4 shows the results of western blotting of the soluble form of the core protein using the anti-GPC3 antibody.
 - Fig. 5 shows the principle of sandwich ELISA using the anti-GPC3 antibody.
 - Fig. 6 is a graph of the standard curve for the GPC3 sandwich ELISA using M6B1 and M18D4.
 - Fig. 7 is a schematic view of the GPC3 structure.
 - Fig. 8 shows combinations of the anti-GPC3 antibodies employed in ELISA.
 - Fig. 9 is a graph of the standard curve for the GPC3 sandwich ELISA system using various combinations of the anti-GPC3 antibodies.
 - Fig. 10 shows the assay results of the ADCC activity of the anti-GPC3 antibody.
 - Fig. 11 shows the assay results of the CDC activity of the anti-GPC3 antibody.

Best Mode for Carrying out the Invention

- **[0125]** The invention is now specifically described in the following Examples. However, the invention is not limited by the Examples.
- [0126] In the Examples described in this specification, the following materials were used.
 - [0127] As expression vectors of the soluble form of GPC3 and the soluble form of the GPC3 core protein, pCXND2 and pCXND3 prepared by integrating the DHFR gene and the neomycin-resistant gene in pCAGGS were used.
 - [0128] DXB11 was purchased from ATCC. For culturing, 5 % FBS (GIBCO BRL CAT# 10099-141, Lot#

AO275242/Minimum Essential Medium Alpha medium (α MEM (+)) (GIBCO BRL CAT# 12571-071)/1 % Penicillin-Streptomycin (GIBCO BRL CAT# 15140-122) was used. For selection of stable cell line of DXB11 expressing each protein, 500 µg/mL Geneticin (GIBCO BRL CAT# 10131-027)/5 % FBS/ α MEM without ribonucleotides and deoxyribonucleotides (GIBCO BRL CAT# 12561-056)(α MEM(-))/PS was used alone or with supplemented with MTX to a final concentration of 25 nM.

[0129] HepG2 was purchased from ATCC and maintained in 10 % FBS/Dulbecco's modified Eagle medium (DMEM) (GIBCO BRL. CAT# 11995-065)/PS.

[0130] The hybridoma was maintained in 10 % FBS/RPMI1640/1 × HAT media supplement (SIGMA CAT# H-0262) /0.5 × BM-Condimed H1 Hybridoma cloning supplement (Roche CAT# 1088947).

Example 1

Cloning and expression analysis of human GPC3 (GPC3) cDNA Cloning of full-length cDNA encoding human glypican 3 (GPC3 hereinafter)

[0131] The full-length cDNA encoding human GPC3 was amplified by PCR, using as a template a first strand cDNA prepared from a colon cancer cell line Caco2 by a general method and Advantage 2 kit (Clontech Cat. No. 8430-1). Specifically, 50 μ l of a reaction solution containing Caco2-derived cDNA of 2 μ l, 1 μ l of a sense primer (SEQ ID NO: 1), 1 μ l of an antisense primer (SEQ ID NO: 2), 5 μ l of Advantage2 10 \times PCR buffer, 8 μ l of dNTP mix (1.25 mM) and 1.0 μ l of Advantage polymerase Mix was subjected to 35 cycles of 94 °C for one minute, 63 °C for 30 seconds and 68 °C for 3 minutes. The amplified product from the PCR (inserted in TA vector pGEM-T easy using pGEM-T Easy Vector System I (Promega Cat No. A1360)) was sequenced using ABI3100 DNA sequencer to confirm that cDNA encoding the full-length human GPC3 was isolated. The sequence represented by SEQ ID NO: 3 indicates the amino acid sequence of the human GPC3 gene, while the sequence represented by SEQ ID NO: 4 indicates the amino acid sequence of human GPC3 protein.

SEQ ID NO: 1: GATATC-ATGGCCGGGACCGTGCGCACCGCGT

SEQ ID NO: 2: GCTAGC-TCAGTGCACCAGGAAGAAGAAGCAC

Expression Analysis of human GPC3 mRNA using GeneChip

[0132] mRNA expression was analyzed in 24 cases with hepatoma lesions (well-differentiated cancer: WD; moderately differentiated cancer: MD; poorly differentiated cancer: PD), 16 hepatoma cases with non-cancer lesions (hepatitis lesion: CH, cirrhosis lesion: LC), 8 cases with normal liver: NL (informed consent acquired; available from Tokyo University, School of Medicine and Saitama Cancer Center), using GeneChipTM UG-95A Target (Affymetrix). Specifically, total RNA was prepared using ISOGEN (Nippon Gene) from the individual tissues, from which 15 μg each of total RNA was used for gene expression analysis according to the Expression Analysis Technical Manual (Affymetrix). [0133] As shown in Fig.1, the mRNA expression level of human GPC3 gene (Probe Set ID: 39350_at) was apparently higher in many of the cases compared with the expression in normal liver tissue, despite the differentiation stages of hepatoma. Furthermore, comparison was made with the mRNA expression of alpha-fetoprotein (Probe Set ID: 40114_at) most commonly used as a diagnostic marker of hepatoma currently. It was shown that even in well-differentiated cancer showing almost no such mRNA expression of alpha-fetoprotein, sufficiently enhanced mRNA expression of GPC3 was observed, and that the ratio of the activation of the mRNA expression of GPC3 was higher. Thus, it is considered that GPC3 detection is useful as a diagnostic method of hepatoma at an early stage.

Example 2

Preparation of anti-GPC3 antibody

Preparation of the soluble form of human GPC3

[0134] As a material for preparing anti-GPC3 antibody, the soluble form of the GPC3 protein lacking the hydrophobic region on the C-terminal side was prepared.

[0135] Using a plasmid DNA containing the complete full-length human GPC3 cDNA supplied from Tokyo University,

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Advanced Technology Institute, a plasmid DNA for expressing the soluble form of the GPC3 cDNA was constructed. PCR was conducted using a downstream primer (5'-ATA GAA TTC CAC CAT GGC CGG GAC CGT GCG C-3') (SEQ ID NO: 5) designed to remove the hydrophobic region on the C-terminal side (564-580 amino acid), and an upstream primer (5'-ATA GGA TCC CTT CAG CGG GGA ATG AAC GTT C-3') (SEQ ID NO.6) with the EcoRI recognition sequence and the Kozak's sequence having been added. The resulting PCR fragment (1711 bp) was cloned in pCXND2-Flag. The prepared expression plasmid DNA was introduced in a CHO cell line DXB11. Selection with 500 µg/mL Geneticin resulted in a CHO line highly expressing the soluble form of GPC3.

[0136] Using a 1700-cm² roller bottle, the CHO line highly expressing the soluble form of GPC3 was cultured at a large scale, and the culture supernatant was collected for purification. The culture supernatant was applied to DEAE Sepharose Fast Flow (Amersham CAT# 17-0709-01), washed, and eluted with a buffer containing 500 mM NaCl. Subsequently, the product was affinity purified using Anti-Flag M2 agarose affinity gel (SIGMA CAT# A-2220) and eluted with 200 µg/mL Flag peptide. After concentration with Centriprep-10 (Millipore Cat# 4304), the Flag peptide was removed by gel filtration with Superdex 200 HR 10/30 (Amersham CAT# 17-1088-01). Finally, the product was concentrated using DEAE Sepharose Fast Flow column, and eluted with PBS (containing 500 mM NaCl) containing no Tween 20 for replacement of the buffer.

Preparation of the soluble form of human GPC3 core protein

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[0137] Using the wild type human GPC3 cDNA as template, cDNA was prepared by assembly PCR, where Ser 495 and Ser 509 were substituted with Ala. A primer was designed in such a fashion that His tag might be added to the C terminus. The resulting cDNA was cloned in pCXND3 vector. The prepared expression plasmid DNA was introduced in a DXB11 line , followed by selection with 500 μ g/mL Geneticin, to obtain the CHO line highly expressing the soluble form of the GPC3 core protein.

[0138] A large scale cultivation was done with a 1700-cm² roller bottle, and the culture supernatant was collected for purification. The supernatant was applied to Q sepharose Fast Flow (Amersham CAT# 17-0510-01), washed, and eluted with a phosphate buffer containing 500 mM NaCl. Subsequently, the product was affinity purified using Chelating Sepharose Fast Flow (Amersham CAT# 17-0575-01), and eluted with a gradient of 10-150 mM imidazole. Finally, the product was concentrated with Q sepharose Fast Flow and eluted with a phosphate buffer containing 500 mM NaCl. [0139] SDS polyacrylamide gel electrophoresis showed a smear-like band of 50 to 300 kDa and a band of about 40 kDa. Fig.2 shows the results of the electrophoresis. GPC3 is a proteoglycan of 69 kDa and with a heparan sulfate-addition sequence at the C terminus. It was considered that the smear-like band corresponds to GPC3 modified with heparan sulfate. The results of amino acid sequencing indicated that the band of about 40 kDa had an origin in the N-terminal fragment. Thus, it was anticipated that GPC3 was more or less cleaved.

[0140] So as to remove antibodies against heparan sulfate in the following screening for hybridoma, the soluble form of the GPC3 core protein where a heparan sulfate-addition signal sequence Ser 495 and Ser 509 were substituted with Ala. CHO cell line highly expressing the protein was prepared as above, and the culture supernatant was affinity purified utilizing the His-tag. SDS polyacrylamide gel electrophoresis showed three bands of 70 kDa, 40 kDa and 30 kDa. Amino acid sequencing indicated that the band of 30 kDa was the C-terminal fragment of GPC3. The C-terminal fragment starts from serine 359 or from valine 375. Thus, it was anticipated that GPC3 received some enzymatic cleavage. The reason why the band of 30 kDa was not observed in the GPC3 of heparan sulfate-added type was that the fragment formed the smear-like band due to the addition of heparan sulfate. It is a novel finding that GPC3 receives enzymatic cleavage at a specific amino acid sequence, but the biological meaning thereof has not yet been elucidated. [0141] The inventors made an assumption on the basis of the results that GPC3 on the membrane even in hepatoma patients would be cleaved and secreted as the soluble form in blood. Compared with AFP as a hepatoma marker, the expression of the gene of GPC3 was found higher in hepatoma patients at earlier stages (Fig. 1). So as to examine the possibility as a novel tumor marker with higher clinical utility than that of AFP, an anti-GPC3 antibody was prepared to construct a sandwich ELISA system as described in Example 2 or below.

Preparation of anti-GPC3 antibody

[0142] Because the homology of human GPC3 with mouse GPC3 is as high as 94 % at the amino acid levels, it was considered that it might be difficult to obtain the anti-GPC3 antibody by the immunization of normal mouse with human GPC3. Thus, MRL/lpr mouse with autoimmune disease was used as an animal to be immunized. Five MRL/lpr mice (CRL) were immunized with the soluble form of GPC3. For the first immunization, the immunogen protein was adjusted to 100 μg/animal and was then emulsified using FCA (Freund's complete adjuvant (H37 Ra), Difco (3113-60), Becton Dickinson (cat# 231131)), which was then subcutaneously administered to the mice. Two weeks later, the protein was adjusted to 50 μg/animal and emulsified with FIA (Freund's incomplete adjuvant, Difco (0639-60), Becton Dickinson (cat# 263910)) for subcutaneous administration to the mice. At one week interval since then, booster was carried out

in total of 5 times. For final booster, the protein was diluted with PBS to 50 µg/animal, which was administered in the caudal vein. By ELISA using an immunoplate coated with the GPC3 core protein, it was confirmed that the serum antibody titer against GPC3 was saturated. A mouse myeloma cell P3U1 and mouse splenocyte were mixed together to allow for cell fusion in the presence of PEG1500 (Roche Diagnostics, cat# 783641). The resulting mixture was inoculated in a 96-well culture plate. From the next day, hybridoma was selected with the HAT medium, the culture supernatant was screened by ELISA. Positive clones were subjected to monocloning by limited dilution method. The resulted monoclone was cultured at an enlarged scale and the culture supernatant was collected. The screening by ELISA was done using the binding activity to the GPC3 core protein as a marker to obtain six clones of an anti-GPC3 antibody with a strong binding potency.

[0143] The antibody was purified using Hi Trap Protein G HP (Amersham CAT# 17-0404-01). The supernatant from the hybridoma culture was applied directly to a column, washed with a binding buffer (20 mM sodium phosphate, pH 7.0) and eluted with an elution buffer (0.1 M glycine-HC1, pH 2.7). The eluate was collected into a tube containing a neutralization buffer (1 M Tris-HC1, pH 9.0) for immediate neutralization. After antibody fractions were pooled, the resulting pool was dialyzed against 0.05 % Tween 20/PBS overnight and for a whole day for buffer replacement. NaN₃ was added to the purified antibody to 0.02 %. The antibody was stored at 4 °C.

Analysis of anti-GPC3 antibody

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[0144] The antibody concentration was assayed by mouse IgG sandwich ELISA using goat anti-mouse IgG (gamma) (ZYMED CAT# 62-6600) and alkali phosphatase-goat anti-mouse IgG (gamma) (ZYMED CAT# 62-6622), along with a commercially available purified mouse IgG1 antibody (ZYMED CAT# 02-6100) as a standard.

[0145] The isotyping of the anti-GPC3 antibody was done with ImmunoPure Monoclonal Antibody Isotyping Kit II (PIERCE CAT# 37502) by the method according to the attached manual. The results of the isotyping indicated that all of the antibodies were of IgG1 type.

[0146] By western blotting using the GPC3 core protein, the epitopes of the anti-GPC3 antibody were classified. The soluble form of the GPC3 core protein was applied to 10 % SDS-PAGE mini (TEFCO CAT# 01-075) at 100 ng/lane for electrophoresis (60 V for 30 min; 120 V for 90 min), and subsequently transferred on Immobilon-P (Millipore CAT# IPVH R85 10) using Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (BIO-RAD) (15 V for 60 min). After the membrane was gently rinsed with TBS-T (0.05 % Tween 20, TBS), the membrane was shaken with 5 % skim milkcontaining TBS-T for one hour (at ambient temperature) or overnight (at 4 °C). After shaking with TBS-T for about 10 minutes, each anti-GPC3 antibody diluted with 1 % skim milk-containing TBS-T to 0.1 to 10 μg/ml was added for onehour with shaking. The membrane was rinsed with TBS-T (10 minutes × three times) and shaken with HRP-anti-mouse IgG antibody (Amersham CAT# NA 931) diluted to 1.1000 with 1 % skim milk-containing TBS-T for one hour, and rinsed with TBS-T (10 minutes × three times). ECL-Plus (Amersham RPN 2132) was used for chromogenic reaction. Hyperfilm ECL (Amersham CAT# RPN 2103K) was used for detection. Fig. 4 shows the results of the western blotting analysis. For the classification, it was determined that the antibody reacting with the band of 40 kDa has an epitope at the N terminus, while the antibody reacting with the band of 30 kDa has an epitope at the C terminus. As antibodies recognizing the N-terminal side, M6B1, M18D4, and M19B11 were obtained. As antibodies recognizing the C-terminal side, M3C11, M13B3, and M3B8 were obtained. The results of the analysis using BIACORE indicated that the KD values of the individual antibodies were in the range of from 0.2 to 17.6 nM.

Example 3

Detection of the secreted form of GPC3

Mouse xenograft model

[0147] 3,000,000 human hepatoma HepG2 cells were transplanted under the abdominal skin in 6-weeks female SCID mice (Fox CHASE C. B-17/Icr-scidJcl, JapanClair) and nudemice (BALB/cAJcl-nu, Japan Clair). 53 days later when tumor was sufficiently formed, whole blood was drawn out from the posterior cava of HepG2-transplanted SCID mice #1, 3, and 4. Plasma was prepared in the presence of EDTA-2Na and aprotinin (Nipro Neotube vacuum blood tube, NIPRO, NT-EA0205) and stored at -20 °C until assay date. In the case of the HepG2-transplanted SCID mouse #2, whole blood was taken 62 days after HepG2 transplantation. In the case of the HepG2-transplanted nude mice #1 and #2, whole blood was taken 66 days after HepG2 transplantation. As a control, plasma was prepared from normal SCID mouse of the same age by the same procedures.

Sandwich ELISA

[0148] So as to detect the secreted form of GPC3 in blood, a sandwich ELISA system of GPC3 was constructed. M6B1 was used as an antibody to be coated in a 96-well plate. M18D4 labeled with biotin was used as an antibody detecting GPC3 bound to M6B1. For chromogenic reaction, AMPAK of DAKO was used for achieving high detection sensitivity.

[0149] A 96-well immunoplate was coated with the anti-GPC3 antibody diluted with a coating buffer (0.1 M NaHCO $_3$, pH 9.6, 0.02 w/v % NaN $_3$) to obtain a concentration of 10 μ g/mL, and incubated at 4 °C overnight. On the next day, the plate was rinsed three times with 300 μ l/well of rinse buffer (0.05 v/v %, Tween 20, PBS) and 200 μ l of dilution buffer (50 mM Tris-HCl, pH 8.1, 1 mM MgCl $_2$, 150 mM NaCl, 0.05 v/v % Tween 20, 0.02 w/v % NaN $_3$, 1 w/v % BSA) was added for blocking. After storage for several hours at ambient temperature or at 4 °C overnight, mouse plasma or the culture supernatant appropriately diluted with a dilution buffer was added and incubated at ambient temperature for one hour. After rinsing with RB at 300 μ l/well three times, the biotin-labeled anti-GPC3 antibody diluted with a dilution buffer to 10 μ g/mL was added, and incubated at ambient temperature for one hour. After rinsing with RB at 300 μ l/well three times, AP-streptoavidin (ZYMED) diluted to 1/1000 with a dilution buffer was added, and incubated at ambient temperature for one hour. After rinsing with the rinse buffer at 300 μ l/well five times, AMPAK (DAKO CAT# K6200) was added for chromogenic reaction according to the attached protocol, and the absorbance was measured with a microplate reader.

[0150] For biotinylation of the antibody, Biotin Labeling Kit (CAT# 1 418 165) of Roche was used. A spreadsheet software GlaphPad PRISM (GlaphPad software Inc. ver. 3.0) was used to calculate the concentration of the soluble form of GPC3 in a sample. Fig.5 shows the principle of the sandwich ELISA in this Example.

[0151] Using the purified soluble form of GPC3, a standard curve was prepared. Consequently, a system with a detection limit of several nanogams/mL could be constructed. Fig.6 shows a standard curve for the GPC3 sandwich ELISA using M6B1 and M18D4. Using the system, an attempt was made to detect the secreted form of GPC3 in the culture supernatant of HepG2 and the serum of a mouse transplanted with human hepatoma HepG2. The secreted form of GPC3 was detected in the culture supernatant of HepG2 and the serum of the mouse transplanted with human hepatoma HepG2, while the secreted form of GPC3 was below the detection limit in the control culture medium and the control mouse serum. On a concentration basis of the purified soluble form of GPC3, the soluble form of GPC3 was at 1.2 μg/mL in the culture supernatant of HepG2 and at 23 to 90 ng/mL in the serum of the mouse (Table 1).

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Assay of the secreted form of GPC3 in the plasma of a mouse transplanted with HepG2 (ng/mL)

	Tumor volume	M6B01(N)-M	M19B11(N)-	M6B1(N)-	M13B3(C)-Bi	M13B3(C)-Bi
	(mm³)	1BD4(N)	M18D4(N)	BioM3C11(C)	oM18D4(N)	
Culture supernatant of HepG2		1190	1736	224	234	₽
HepG2-transplanted SCID mouse #1	2022	65.4	76.9	<10	<10	<10
HepG2-transplanted SCID mouse #2	1706	71.7	94.8	<10	<10	<10
HepG2-transplanted SCID mouse #3	2257	90.3	113.9	<10	<10	<10
HepG2-transplanted SCID mouse #4	2081	87.3	107.3	<10	15.0	<10
HepG2-transplanted nude mouse #1	1994	58.7	53.6	19.7	35.5	102.2
HepG2-transplanted nude mouse #2	190 & 549	22.9	33.6	<10	11.5	40.6
Normal SCID mouse #1	0	<10	<10	<10	<10	<10
Normal SCID mouse #2	0	<10	<10	<10	<10	<10
Normal SCID mouse #3	0	<10	<10	<10	<10	<10

Structure of secreted form of GPC3

[0152] It was examined whether or not the blood-secreted GPC3 has the structure of the N-terminal fragment as preliminarily assumed. In case that the secreted form of GPC3 was the N-terminal fragment, it is considered that the secreted form of GPC3 will not be detected by sandwich ELISA with a combination of an antibody recognizing the N terminus and an antibody recognizing the C terminus. Using three types of each antibody recognizing the N-terminal fragment and each antibody recognizing the C-terminal fragment, sandwich ELISA systems with various combinations were constructed. Fig.7 shows the structure of the secreted form of GPC3 and Fig.8 shows combinations of the antibodies. Fig.9 shows a standard curve of the sandwich ELISA. Table 1 shows the assay results. As shown in Table 1, the secreted form of GPC3 was detected at higher values in the culture supernatant of HepG2 and the serum of a mouse transplanted with human hepatoma HepG2 with combinations of antibodies recognizing the N-terminal fragment, while it was detected below the detection limit in many samples from the mice with the systems containing antibodies recognizing the C-terminal fragment. Thus, it was anticipated that the secreted GPC3 was possibly detected at a high sensitivity by using an antibody against the amino acid sequence comprising the amino acid residue 1 to the amino acid residue 374 of GPC3.

Example 4

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20 Preparation of anti-GPC3 mouse-human chimera antibody

[0153] Using total RNA extracted from a hybridoma producing an antibody capable of binding to human GPC3 (human GPC3-antibody recognizing C-terminus: M3C11, M1E07; human GPC3-antibody recognizing N terminus: M19B11, M18D04, M5B09, M10D02), the cDNA of variable region of the antibody was amplified by RT-PCR. The total RNA was extracted from the hybridoma of 1×10^7 cells, using RNeasy Plant Mini Kits (manufactured by QIAGEN). Using 1 μ g of the total RNA and also using SMART RACE cDNA Amplification Kit (manufactured by CLONTECH), a synthetic oligonucleotide MHC-IgG1 (SEQ ID NO:7) complementary to the mouse IgG1 constant region sequence or a synthetic oligonucleotide kappa (SEQ ID NO:8) complementary to the nucleotide sequence of the mouse κ chain constant region, a 5'-terminal fragment of the gene was amplified. The reverse-transcription was done at 42 °C for one hour and 30 minutes. 50 μ l of the PCR solution contained 5 μ l of 10 \times Advantage 2 PCR Buffer, 5 μ l of 10 \times Universal Primer A Mix, 0.2 mM dNTPs (dATP, dGTP, dCTP, dTTP), 1 μl of Advantage 2 Polymerase Mix (all manufactured by CLONTECH), 2.5 μl of the reverse-transcription product, and 10 pmole of the synthetic oligonucleotideMHC-lgG1 or kappa. After the initial temperature at 94 °C for 30 seconds, a cycle of 94 °C for 5 seconds and 72 °C for 3 minutes was repeated five times; a cycle of 94 °C for 5 seconds, 70 °C for 10 seconds and 72 °C for 3 minutes was repeated five times; and a cycle of 94 °C for 5 seconds, 68 °C for 10 seconds and 72 °C for 3 minutes was repeated 25 times. Finally, the reaction product was heated at 72 °C for 7 minutes. After the individual PCR products were purified from agarose gel using QIAquick Gel Extraction Kit (manufactured by QIAGEN), the products were cloned in pGEM-T Easy vector (manufactured by Promega), and the nucleotide sequence was determined.

[0154] Then, the sequences of the variable regions of the H chain and L chain were linked to the constant regions of the human H chain and L chain. PCR was done using a synthetic oligonucleotide complementary to the 5'-terminal nucleotide sequence of the H chain variable region of each antibody and having the Kozak's sequence and a synthetic oligonucleotide complementary to the 3'-terminal nucleotide sequence and having an Nhel site. The resulting PCR products were cloned in a pB-CH vector with the human IgG1 constant region inserted in pBluescript KS+ vector (manufactured by TOYOBO). The mouse H chain variable region and the human H chain (γ1 chain) constant region are liked together via the Nhel site. The prepared H chain gene fragment was cloned in an expression vector pCXND3. The scheme of the construction of the vector pCXND3 is described below. So as to divide the gene encoding the antibody H chain and the vector sequence from DHFR-ΔE-rvH-PM1-f (see WO 92/19759), the vector was digested at the restriction enzyme EcoRI/Smal sites to recover only the vector sequence. Subsequently, the vector sequence was cloned in EcoRI-NotI-BamHI adaptor (manufactured by Takara Shuzo Co., Ltd.). This vector was designated as pCHO1. A region from pCHO1 expressing the DHFR gene was cloned in pCXN at the restriction enzyme HindIII site (Niwa et al., Gene 1991: 108: 193-200). The resulting vector was designated as pCXND3. The nucleotide sequences of the H chains of the anti-GPC3 mouse-human chimera antibodies (M3C11, MIE07, M19B11, M18D04) contained in each plasmid are shown as SEQ ID NOS: 9, 11, 13 and 15, respectively. The amino acid sequences thereof are shown as SEQ ID NOS: 10, 12, 14, and 16, respectively. Additionally, PCR was done using a synthetic oligonucleotide complementary to the 5'-terminal nucleotide sequence of the L chain variable region of each antibody and having the Kozak's sequence and a synthetic oligonucleotide complementary to the 3'-terminal nucleotide sequence and having a BsiWI site. The resulting PCR products were cloned in a pB-CL vector, where the human kappa chain constant region was preliminarily inserted in pBluescript KS+ vector (manufactured by TOYOBO). The human L chain variable region and

the constant region were linked together via the BsiWI site. The prepared L chain gene fragment was cloned in an expression vector pUCAG. The vector pUCAG is a vector prepared by digesting pCXN (Niwa et al., Gene 1991: 108: 193-200) with restriction enzyme BamHI to obtain a 2.6-kbp fragment, which is then cloned into the restriction enzyme BamHI site of pUC19 vector (manufactured by TOYOBO). The nucleotide sequences of the L chains of the anti-GPC3 mouse-human chimera antibodies (M3C11, M1E07, M19B11, M18D04) contained in each plasmid are shown as SEQ ID NOS: 17, 19, 21 and 23, respectively. The amino acid sequences thereof are shown as SEQ ID NOS: 18, 20, 22 and 24, respectively.

[0155] So as to prepare an expression vector of the anti-GPC3 mouse-human chimera antibody, a gene fragment obtained by digesting the pUCAG vector having the L chain gene fragment inserted therein with restriction enzyme HindIII (manufactured by Takara Shuzo Co., Ltd.) was cloned into the restriction enzyme HindIII cleavage site of pCXND3 having the H chain gene inserted therein. The plasmid will express the neomycin-resistant gene, the DHFR gene and the anti-GPC3 mouse-human chimera antibody gene in animal cells.

[0156] A CHO-based cell line for stable expression (DG44 line) was prepared as follows. The gene was introduced by electroporation method using Gene PulserII (manufactured by Bio Rad). 25 μ g of each expression vector of the anti-GPC3 mouse-human chimera antibody and 0.75 ml of CHO cells (1 \times 10⁷ cells/ml) suspended in PBS were mixed together, and cooled on ice for 10 minutes, which was then transferred into a cuvette and received a pulse at 1.5 kV and 25 μ FD. After a recovery time at ambient temperature for 10 minutes, the cells treated by the electroporation were suspended in 40 mL of a CHO-S-SFMII culture medium (manufactured by Invitrogen) containing 1 \times HT supplement (manufactured by Invitrogen). A 50-fold dilution was prepared using the same culture medium, and added at 100 μ l/ well in a 96-well culture plate. After culturing in a CO₂ incubator (5 % CO₂) for 24 hours, Geneticin (manufactured by Invitrogen) was added to 0.5 mg/mL, and continued cultivation for 2 weeks. The IgG in the culture supernatant from the wells of colonies of a Geneticin resistance transformant cell was assayed by the following concentration assay method. A cell line with high productivity was expanded at an enlarged scale. The cell line stably expressing the anti-GPC3 mouse-human chimera antibody was cultured in a large-scale culturing and the culture supernatant was collected

[0157] The IgG concentration in the culture supernatant was assayed by human IgG sandwich ELISA using Goat Anti-human IgG (manufactured by BIOSORCE) and Goat Anti-human IgG alkaline phosphatase conjugated (manufactured by BIOSORCE) and compared with the commercially available purified human IgG (manufactured by Cappel). [0158] Each anti-GPC3 mouse-human chimera antibody was purified using Hi Trap Protein G HP (manufactured by Amersham). A culture supernatant of a CHO cell line producing the anti-GPC3 mouse-human chimera antibody was directly applied to a column and eluted with elution buffer (0.1 M glycine-HC1, pH 2.7). Eluate was collected into a tube containing a neutralization buffer (1 M Tris-HC1, pH 9.0) for immediate neutralization. Antibody fractions were pooled and dialyzed against 0.05% Tween 20/PBS overnight and for a whole day to replace the buffer. NaN₃ was added to the purified antibody to 0.02 % and stored at 4 °C.

Example 5

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Preparation of a CHO cell line stably expressing the full length GPC3

[0159] Human GPC3 cDNA was obtained by digesting pGEM-T Easy vector with the full-length human GPC3 cDNA cloned therein with restriction enzyme EcoRI (manufactured by Takara Shuzo Co., Ltd.) and cloned in an expression vector pCOS2. The scheme of the construction of the vector pCOS2 is described below. So as to divide the gene of the antibody H chain of DHFR-ΔE-rvH-PM1-f (see WO 92/19759) from the vector, the vector was digested at the restriction enzyme EcoRI/Smal sites, to recover only the vector sequence. Subsequently, the vector sequence was cloned in EcoRI-NotI-BamHI adaptor (manufactured by Takara Shuzo Co., Ltd.). This vector was designated as pCHO1. A region from pCHO1 expressing the DHFR gene was removed, into which the sequence of the neomycin resistant gene in HEF-VH-gy1 (Sato et al., Mol. Immunol. 1994: 31: 371-381) was inserted. The vector was designated as pCOS2. [0160] A cell line stably expressing the full-length human GPC3 was prepared as follows. 10 µl of the full-length human GPC3 gene-expressing vector and 60 µl of SuperFect (manufactured by QIAGEN) were mixed together, to form a complex, which was then added to a CHO cell line DXB11 to introduce the gene. After culturing in a CO2 incubator (5 % CO₂) for 24 hours, αMEM (manufactured by GIBCO BRL) containing Geneticin (manufactured by Invitrogen) to a final concentration of 0.5 mg/mL and 10 % FBS (manufactured by GIBCO BRL) was used to start selection. The resulting Geneticin-resistant colonies were collected and cell cloning was done by limited dilution method. Individual cell clones were solubilized to confirm the expression of the full-length human GPC3 by western blotting using the anti-GPC3 antibody. A cell strain stably expressing human GPC3 was obtained.

Example 6

ADCC assay using PBMC derived from human peripheral blood

(1) Preparation of human PBMC

[0161] Peripheral blood was collected from normal subjects with heparinized syringes, and diluted to 2 fold with PBS (-), and overlaid on FicoII-Paque™ PLUS (Amersham Pharmacia Biotech AB). This was centrifuged (500 × g, 30 minutes, 20 °C), and collected the intermediate layer as a mononuclear cell fraction. After rinsing three times, the resulting fraction was suspended in 10 % FBS/RPMI to prepare a human PBMC solution.

(2) Preparation of target cell

[0162] HepG2 cell cultured in 10 % FBS/RPMI 1640 culture medium was detached from the dish using trypsin-EDTA (Invitrogen Corp), divided in each well at 1×10^4 cells/well in a U-bottom 96-well plate (Falcon), and cultured for 2 days. After culturing, 5.55 MBq of chromium-51 was added and the cells were incubated in a 5 % CO₂ gas incubator at 37 °C for one hour. The resulting cells were rinsed once with the culture medium, to which 50 μ l of 10 % FBS/RPMI 1640 culture medium was added to prepare a target cell.

(3) Chromium release test (ADCC activity)

[0163] $50~\mu$ l of an antibody solution prepared to each concentration was added to the target cell on ice for 15 minutes. Subsequently, 100 μ l of a human PBMC solution was added (5 \times 10⁵ cells/well), and incubated in a 5 % CO₂ gas incubator at 37 °C for 4 hours. After incubation, the plate was centrifuged and the radioactivity in 100 μ l of the culture supernatant was counted with a gamma counter. The specific chromium release ratio was determined by the following formula:

Specific chromium release ratio (%) = $(A-C) \times 100/(B-C)$

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[0164] "A" represents the mean radioactivity value (cpm) in each well; "B" represents the mean radioactivity value (cpm) in a well where 100 μ l of aqueous 2 % NP-40 solution (Nonidet P-40, Code No. 252-23, Nakarai Tesque) and 50 μ l of 10 % FBS/RPMI culture medium were added to the target cell; and "C" represents the mean radioactivity value (cpm) in a well where 150 μ l of 10 % FBS/RPMI culture medium was added to the target cell. The test was done in triplicate to calculate the mean of the ADCC activity (%) and the standard error.

[0165] The results are shown in Fig.10. Among the six types of anti-GPC3 chimera antibodies, the antibodies ch. M3C11 and ch.M1E07 recognizing the C terminus exerted the ADCC activity, while the antibodies ch. M19B11, ch. M18D04, ch. M5E09 and ch. M10D02 recognizing the N terminus hardly exerted the ADCC activity. The above results indicate that the ADCC activities of the chimera antibodies depend on the recognition sites of the antibodies. Further, it was expected that the antibodies recognizing the C terminus of GPC3 were possibly useful in clinical applications since the antibodies recognizing the C terminal sides from the cleavage sites exerted the ADCC activity.

Example 7

- 45 Assay of compliment-dependent cytotoxic activity (CDC activity)
 - (1) Preparation of human albumin veronal buffer (HAVB)

[0166] 12.75 g of NaCl (superior grade; Wako Pure Chemical Industries, Ltd.), 0.5625 g of Na-barbital (superior grade; Wako Pure Chemical Industries, Ltd.), and 0.8625 g of barbital (superior grade; Wako Pure Chemical Industries, Ltd.) were dissolved in Milli Q water to 200 mL, and autoclaved (121 °C, 20 minutes). 100 mL of autoclaved warm Milli Q water was added. Then, it was confirmed that the resulting mixture was at pH 7.43 (pH 7.5 recommended). This was defined as 5 × Veronal Buffer. 0.2205 g of CaCl₂-2H₂O (superior grade; Wako Pure Chemical Industries, Ltd.) was dissolved in 50 mL of Milli Q water to 0.03 mol/L. The resulting solution was defined as CaCl₂ solution. 1.0165 g of MgCl₂-6 H₂O (superior grade; Wako Pure Chemical Industries, Ltd.) was dissolved in 50 mL of Milli Q water to 0.1 mol/L. The resulting solution was defined as MgCl₂ solution. 100 mL of 5 × Veronal Buffer, 4 mL of human serum albumin (Buminate^R 25 %, 250 mg/mL of human serum albumin concentration, Baxter), 2.5 mL of the CaCl₂ solution, 2.5 mL of the MgCl₂ solution, 0.1 g of KCl (superior grade; Wako Pure Chemical Industries, Ltd..), and 0.5 g of glucose

(D (+)-glucose, anhydrous glucose, superior grade; Wako Pure Chemical Industries, Ltd.) were dissolved in Milli Q water to 500 mL. This was defined as HAVB. After filtration and sterilization, the resulting solution was stored at a set temperature of 5 °C.

(2) Preparation of target cell

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[0167] CHO cell expressing GPC3 on the cell membrane as prepared in Example 4 was cultured in alpha-MEM nucleic acid (+) culture medium (GIBCO) supplemented with 10 % FBS and 0.5 mg/mL Geneticin (GIBCO), detached from the dish using a cell dissociation buffer (Invitrogen Corp), and divided at 1 \times 10⁴ cells/well in each well of a 96-well flat bottom plate (Falcon), for culturing for 3 days. After culturing, 5.55 MBq of chromium-51 was added, and incubated in a 5 % CO₂ gas incubator at 37 °C for one hour. The resulting cell was rinsed twice with HAVE, to which 50 μ l of HAVE was added to prepare a target cell.

(3) Chromium release test (CDC activity)

[0168] Each chimera antibody was diluted with HAVE to prepare an antibody solution of 40 µg/mL. The antibody solution was added in a 50 µl-portion to the target cell, which was then left on ice for 15 minutes. Subsequently, baby rabbit compliment (Cedarlane) diluted with HAVB was added in 100 µl portions to each well to a final concentration of 30 % (final antibody concentration of 10 µg/mL), and incubated in a 5 % CO₂ gas incubator at 37 °C for 90 minutes. After centrifugation of the plate, a 100-µl portion of the supernatant was recovered from each well, and the radioactivity was measured with a gamma counter. The specific chromium release ratio was determined by the following formula:

Specific chromium release ratio (%) = $(A-C) \times 100/(B-C)$

[0169] "A" represents the mean radioactivity value (cpm) in each well; "B" represents the mean radioactivity value (cpm) in a well where $100 \,\mu$ l of aqueous 2 % NP-40 solution (Nonidet P-40, Code No. 252-23, Nakarai Tesque) and 50 $\,\mu$ l of HAVB were added to the target cell; and "C" represents the mean radioactivity value (cpm) in a well where 150 $\,\mu$ l of HAVE was added to the target cell. The test was done in triplicate to calculate the mean of the CDC activity (%) and the standard error.

[0170] The results are shown in Fig.11. Among the six types of the anti-GPC3 chimera antibodies, the antibodies ch.M3C11 and M1E07 recognizing the C terminus exerted the CDC activity, while the antibodies ch. M19B11, ch. M18D04, ch. M5E09 and ch. M10D02 recognizing the N terminus exerted low CDC activities. The above results indicate that the CDC activities of the chimera antibodies depend on the recognition sites of the antibodies. Further, it was expected that the antibodies recognizing the C terminus of GPC3 were possibly useful in clinical applications since the antibodies recognizing the C terminal sides from the cleavage sites exerted the CDC activity.

Industrial Applicability

[0171] As shown in the Examples, it was suggested such that a portion of GPC3 highly expressed in hepatoma cells may exist as a secreted form in blood. Because the gene expression of GPC3 is observed at an earlier stage than that of AFP, a hepatoma marker, GPC3 detection is expected to be useful for cancer diagnosis. It is observed that GPC3 is expressed in cancer cell lines other than hepatoma cell lines, such as lung cancer, colon cancer, breast cancer, prostate cancer, pancreatic cancer and lymphoma. Accordingly, GPC3 is possibly applicable to the diagnosis of cancers other than hepatoma.

[0172] Additionally, it is also suggested that a secreted form of GPC3 in blood predominantly comprises the N-terminal fragment of about 40 kDa, which is observed in the soluble form of the GPC3 core protein. This indicates that antibodies recognizing the N-terminal fragment are useful as the antibody for use in such diagnosis. In addition, if antibodies recognizing the C-terminal fragment with the ADCC activity and/or the CDC activity are used for treating hepatoma, the antibodies can efficiently reach hepatoma cell without being trapped by the secreted form of GPC3 present in blood. Thus, such antibodies are useful as agents for disrupting cancer cells and as anti-cancer agents.

[0173] The contents of all the publications listed in this specification are entirely included in the specification. Additionally, a person skilled in the art will readily understand that various modifications and variations of the invention are possible without departure from the technical scope and inventive range described in the attached claims. It is intended that the invention also encompasses such modifications and variations.

SEQUENCE LISTING

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			tgc														1413
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	nia	nıa	піб	лоп	440	ine t	Буз	no n	UII	445	Non	ւշս	1113	Giu	450	Буб	
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	LCU	565	1111	361	MC I		570	561	141	741	CYS	575	rne	Luc	Leu	rai	
50		300										J 1 U					
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40	GIy		Phe	Pro	Lys	Leu		Me t	Thr	Gin	Val		Lys	Ser	Leu	GIn
		210			n :		215					220		a 1	T 7 1	T1.
45		Thr	Arg	He	Phe		Gln	Ala	Leu	ASD		Gly	116	GIU	Val	
	225		mı		11.	230		D.I.	0	Υ	235		0.1		W. 4	240
	Asn	Thr	Thr	Asp		Leu	Lys	Phe	5e r		ASP	Cys	Gly	Arg		Leu
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	Pr	о Суз	s Gly	Gly	Ty:	Cys	s Asi	ı Va	l Va	l Me	t Glr	Gl	у Су	s Me	t Ala	Gly
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30											CCC						1056
	C y S	r A 2	141	340	изп	LyS	ніа	ren	345	Ald	Pro	116	GILL	350	1111	116	
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	305					310					315					320

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5	6,5	j				70					75					80	
	cap	, 220	ttc	a a o	ልቦር	220	grr	ara	ftor	act	σta	gar	222	tee	tee	300	288
10			Phe														200
	OIL	. Буо	1110	цуз	85	Lys	піа	1111	DC II			ΛSÞ	газ	261			
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15		~~-	.			- 4 -			_ 4 _					1.1			000
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				100					105					110			
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30				aaa Lys											1104
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55							-								

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			tcc ttc ttc ctc tac	γ
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20	Pro	G1n 370	Val	Tyr	Thr	Leu	Pro 375	Pro	Ser	Arg	Asp	G1 u 380	Leu	Thr	Lys	Asn
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05	gtc ca	o tot	σασ	oto	cag	rta	øtø	ភ ១ ស	tet	ውውው	០០១	gar	tta	øtø	ลลฐ	96
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	, al di	и оуз	20	141	OIL	LCu	741	25	JUL	GIY	Uly	пор	30	741	L J 5	
40			20					40					00			
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	Pro Gl															
45	110 01	35	1111	bcu	Буз	DC u	40	0,5	· · · · · ·	пп	JUI	45	501	****		
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	Thr	Phe	Pro	Ala	Val	Leu	Gln	Ser	Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser	
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	1				5					10)				15	
	. 17-1	C1-	C	01	₩ - 1	01	T	V - 1	Q1	· C · ·	0.1	01		T.	11 1	
10	Val	GIII	. Cys	20		GIN	Leu	yaı	25	Ser	GIY	Gly	ASP	Leu 30		Ly:
				20					40					อบ		
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	Ser		Tyr	Ala	Met	Ser		Val	Arg	Gln	Thr		Glu	Lys	Arg	Leu
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					. 85					90					95	
35	Th	T		T	01	и ,		n -	τ	4	0	0.1		T.I		D.
	Inr	Leu	lyr	100	GIN	Met	ASI	ser	105	Arg	ser	Glu	ASP	110	Ala	Pne
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	Lve	Clay	Dma	C 0. =	Vol	Dha	Dro	Lou	1 I a	D = 4	C	C = =	Tern	Sor.	ፐ ክ -	Ca-
55	LYS	ΩIΆ	rro	ser	lsv	rne	LIO	Leu	Ala	Pro	ser	зег	r A 2	ser	TÜL	196

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	σtσ	асо	art ar	tea	taa	220	tra	σσς	gee	ctg	acc	ვთი	aar	or to	cac	acc	576
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50										Phe							010
	ren	rcu	ΩΙŊ		110	261	141	TITE		1 11 6	110	t I O	гуэ		n 19	ιτοħ	
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55																	

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	0.00	000	taa	o a t	~ t ~	a to		a t a	. • •		~ t ^						1000
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	30											٠					,
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•	35	C1	T	DI	T	0.1			,		mı		** ,			77 1	
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					an
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15		ag tgt ln Cys														96
	cct g Pro G	ga gga ly Gly 35	tcc Ser	ctg Leu	aaa Lys	ctc Leu	tcc Ser 40	tgt Cys	gca Ala	gcc Ala	tct Ser	gga Gly 45	ttc Phe	act Thr	ttc Phe	144
20	Ser A	gc tat rg Tyr 50														192
		gg gtc rp Val														240
25		ct gtg hr Val														288
	Thr L	tg cac eu His	Leu 100	Gln	Met	Arg	Ser	Leu 105	Arg	Ser	Glu	Asp	Thr 110	Ala	Leu	336
30	tat ta Tyr T	ac tgt yr Cys 115	gta Val	aga Arg	cag Gln	ggg Gly	ggg Gly 120	gct Ala	tac Tyr	tgg Trp	ggc Gly	caa Gln 125	ggg Gly	act Thr	ctg Leu	384
	Val T	ct gtc hr Val 30														432
35	Ala P: 145	cc tcc ro Ser	Ser	Lys	Ser 150	Thr	Ser	Gly	Gly	Thr 155	Ala	Ala	Leu	Gly	Cys 160	480
	Leu V	tc aag al Lys	Asp	Tyr 165	Phe	Pro	Glu	Pro	Val 170	Thr	Val	Ser	Trp	Asn 175	Ser	528
40	Gly A	cc ctg la Leu	Thr 180	Ser	Gly	Val	His	Thr 185	Phe	Pro	Ala	Val	Leu 190	Gln	Ser	576
45	Ser G	ga ctc ly Leu 195	Tyr	Ser	Leu	Ser	Ser 200	Val	Val	Thr	Val	Pro 205	Ser	Ser	Ser	624
	Leu G	gc acc ly Thr 10	Gln	Thr	Tyr	Ile 215	Cys	Asn	Val	Asn	His 220	Lys	Pro	Ser	Asn	672
50	Thr L; 225	ag gtg ys Val	Āsp	Lys	Lys 230	Val	Glu	Pro	Lys	Ser 235	Cys	Asp	Lys	Thr	His 240	720
	Thr C	gc cca ys Pro	Pro	Cys 245	Pro	Ala	Pro	Glu	Leu 250	Leu	Gly	Gly	Pro	Ser 255	Val	768
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                                      280
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            Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys
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                                                        300
            aca aag ccg cgg gag gag cag tac aac agc acg tac cgt gtg gtc agc
            Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser
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                                             315
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                                                                               1008
            Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys
                                               330
                                                                               1056
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            Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile
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                                           345
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            tcc aaa gcc aaa ggg cag ccc cga gaa cca cag gtg tac acc ctg ccc
            Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro
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                                    360
                                                            365
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            Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn
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            ggg cag ccg gag aac aac tac aag acc acg cct ccc gtg ctg gac tcc
            Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser
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                          405
                                             410
                                                                               1296
            gac ggc tcc ttc ttc ctc tac agc aag ctc acc gtg gac aag agc agg
            Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg
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            tgg cag cag ggg aac gtc ttc tca tgc tcc gtg atg cat gag gct ctg
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            Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu
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45
            Val Gln Cys Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Lys
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                                            25
            Pro Gly Gly Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
                    3.5
                                        40
                                                             45
            Ser Arg Tyr Ala Met Ser Trp Val Arg Gln Ile Pro Glu Lys Ile Leu
                                    55
                                                      60
50
            Glu Trp Val Ala Ala Ile Asp Ser Ser Gly Gly Asp Thr Tyr Tyr Leu
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                               70
            Asp Thr Val Lys Asp Arg Phe Thr Ile Ser Arg Asp Asn Ala Asn Asn
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                                                90
            Thr Leu His Leu Gln Met Arg Ser Leu Arg Ser Glu Asp Thr Ala Leu
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Tyr Tyr Cys Val Arg Gln Gly Gly Ala Tyr Trp Gly Gln Gly Thr Leu
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         Val Thr Val Ser Ala Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
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                    135
           130
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         Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys
                       150 155
         145
         Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser
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                     165 170
         Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser
                                   185 190
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                   180
         Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser
              195 200 205
         Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn
                   215
                                     220
         Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His
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                       230
                                         235
         Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val
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                                                     255
                    245
         Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr
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                260
         Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu
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                       280
                                                285
              275
         Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys
                                           300
            290
                           295
         Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser
                       310 315
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         Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys
                    325 330 335
         Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile
                   340 345 350
         Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro
                355
                                360
                                                 365
30
         Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
                            375
                                             380
         Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn
                         390
                                         395
         Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser
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         Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg
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                  420
         Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu
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10			tac	tac Tyr				gtg	_		_		gaa	-	_		192
		tgg		gga Gly			aat					ggt					240
15	cag	-		aag Lys	-	_	-		_		_	-				-	288
		-		atg Met 100	_		_	-	_				-		_	-	336
20				gca Ala													384
				caa Gln			_	_		_		-	_	_		_	432
25			_	gtc Val			_	-				_	-				480
				gcc Ala													528
30			-	tcg Ser 180	-				-	_		_					576
			_	gtc Val		_								_	-		624
35				ccc Pro		-	_				_				-		672
				aag Lys						_	-	_		_			720
40			-	gac Asp					_		_	_		_		_	768
45				gga Gly 260													816
	Thr	Leu	Met 275	atc Ile	Ser	Arg	Thr	Pro 280	Glu	Val	Thr	Cys	Val 285	Val	Val	Asp	864
50	gtg Val	agc Ser 290	cac His	gaa Glu	gac Asp	cct Pro	gag Glu 295	gtc Val	aag Lys	ttc Phe	aac Asn	tgg Trp 300	tac Tyr	gtg Val	gac Asp	ggc Gly	912
				cat His		_	_			_		-		-			960
55	_			cgt Arg		-	_	_			_	_		_	-		1008

```
ctg aat ggc aag gag tac aag tgc aag gtc tcc aac aaa gcc ctc cca
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                                           345
            ged eed ate gag aaa ace ate tee aaa ged aaa ggg eag eed ega gaa
                                                                            1104
            Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu
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                                                                            1152
            cca cag gtg tac acc ctg ccc cca tcc cgg gat gag ctg acc aag aac
            Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn
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                                  375
                                                      380
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            Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile
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            385
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            Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr
                                            410
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                                                                            1296
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            Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys
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                                         425
                                                            430
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            Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys
                   435
                                      440
                                                   445
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            tcc gtg atg cat gag gct ctg cac aac cac tac acg cag aag agc ctc
                                                                            1392
            Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu
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            Ser Leu Ser Pro Gly Lys
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                                           25
            Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ser Pho
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                    35
                                       40
            Thr Gly Tyr Tyr Met His Trp Val Lys Gln Ser Pro Glu Lys Ser Leu
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            Glu Trp Ile Gly Glu Ile Asn Pro Ser Thr Gly Gly Thr Thr Tyr Asn
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                                                   75
            Gln Lys Phe Lys Ala Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser
45
                            85
                                               90
            Thr Ala Tyr Met Gln Leu Lys Ser Leu Thr Ser Glu Asp Ser Ala Val
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                                         105
                                                              110
            Tyr Tyr Cys Ala Arg Arg Gly Gly Leu Thr Gly Thr Ser Phe Phe Ala
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                               120
                                                        125
            Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ala Ala Ser Thr Lys
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                                  135
                                                    140
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            Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly
                            150 155
            Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro
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                           165
            Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr
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180
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                             215
                                               220
         Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro
          225 230
                                          235
          Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu
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                                     250
                                                       255
          Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp
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                  260 · 265
          Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp
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          Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly
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                  340 345 350
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          Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu
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                                 360
                                                  365
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                                              380
         Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile
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                         390
                                           395
         Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr
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                                      410
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                                    425
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          1 5
                               10
50
                                                                  96
          gtc cag tgt gag gtg cag ctg gtg gag tct ggg gga gac tta gtg aag
         Val Gln Cys Glu Val Gln Leu Val Glu Ser Gly Gly Asp Leu Val Lys
                                     25
          cct gga ggg acc ctg aaa ctc tcc tgt gca gcc tct gga tcc act ttc
          Pro Gly Gly Thr Leu Lys Leu Ser Cys Ala Ala Ser Gly Ser Thr Phe
55
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5	gag Glu 65	tgg Trp	gtc Val	gca Ala	gcc Ala	att Ile 70	gat Asp	agt Ser	aat Asn	gga Gly	ggt Gly 75	acc Thr	acc Thr	tac Tyr	tat Tyr	cca Pro 80	240
	Āsp	Thr	Met	Lys	gac Asp 85	Arg	Phe	Thr	Ile	Ser 90	Arg	Asp	Asn	Ala	Lys 95	Asn	288
10					caa Gln												336
	Tyr	His	Cys 115	Thr	aga Arg	His	Asn	Gly 120	Gly	Tyr	Glu	Asn	Tyr 125	Gly	Trp	Phe	384
15	gct Ala	tac Tyr 130	tgg Trp	ggc Gly	caa Gln	ggg Gly	act Thr 135	ctg Leu	gtc Val	act Thr	gtc Val	tct Ser 140	gca Ala	gct Ala	agc Ser	acc Thr	432
20	aag Lys 145	ggc Gly	cca Pro	tcg Ser	gtc Val	ttc Phe 150	ccc Pro	ctg Leu	gca Ala	ccc Pro	tcc Ser 155	tcc Ser	aag Lys	agc Ser	acc Thr	tct Ser 160	480
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25	ccg Pro	gtg Val	acg Thr	gtg Val 180	tcg Ser	tgg Trp	aac Asn	tca Ser	ggc Gly 185	gcc Ala	ctg Leu	acc Thr	agc Ser	ggc Gly 190	gtg Val	cac His	576
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30	gtg Val	gtg Val 210	acc Thr	gtg Val	ccc Pro	tcc Ser	agc Ser 215	agc Ser	ttg Leu	ggc Gly	acc Thr	cag Gln 220	acc Thr	tac Tyr	atc Ile	tgc Cys	672
					aag Lys												720
35					gac Asp 245												768
					gga Gly												816
40	gac Asp	acc Thr	ctc Leu 275	atg Met	atc Ile	tcc Ser	cgg Arg	acc Thr 280	cct Pro	gag Glu	gtc Val	aca Thr	tgc Cys 285	gtg Val	gtg Val	gtg Val	864
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	aac Asn	agc Ser	acg Thr	tac Tyr	cgt Arg 325	gtg Val	gtc Val	agc Ser	gtc Val	ctc Leu 330	acc Thr	gtc Val	ctg Leu	cac His	cag Gln 335	gac Asp	1008
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	cca Pro	gcc Ala	ccc Pro 355	atc Ile	gag Glu	aaa Lys	acc Thr	atc Ile 360	tcc Ser	aaa Lys	gcc Ala	aaa Lys	ggg Gly 365	cag Gln	ccc Pro	cga Arg	1104
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                                              395
          atc gcc gtg gag tgg gag agc aat ggg cag ccg gag aac aac tac aag
                                                                        1248
          Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys
                                   410
                                                     415
          acc acg cct ccc gtg ctg gac tcc gac ggc tcc ttc ttc ctc tac agc
                                                                        1296
10
          Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser
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          Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser
              435
                                   440
                                                                        1392
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          Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser
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          Pro Gly Gly Thr Leu Lys Leu Ser Cys Ala Ala Ser Gly Ser Thr Phe
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          Ser Asn Tyr Ala Met Ser Trp Val Arg Gln Thr Pro Glu Lys Arg Leu
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          Glu Trp Val Ala Ala Ile Asp Ser Asn Gly Gly Thr Thr Tyr Tyr Pro
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                                               75
          Asp Thr Met Lys Asp Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn
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          Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ser Glu Asp Thr Ala Phe
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                                      105
                                                          110
          Tyr His Cys Thr Arg His Asn Gly Gly Tyr Glu Asn Tyr Gly Trp Phe
                                 120
                                                    125
               115
          Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ala Ala Ser Thr
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                                                 140
           130
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          Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
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          145
          Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu
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180 185 190
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          Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser
195 200 205
          Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys
                             215
                                                220
          Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu
55
```

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                              265
                      260
           Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val
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                  275
                                  280
           Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp
                                 295
                                                   300
           Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr
                                                         320
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Claims

10

15

- 1. An antibody against an N-terminal peptide of GPC 3.
- The antibody claimed in Claim 1 wherein the N-terminal peptide of GPC 3 is a secreted form of a peptide found in blood.
 - 3. The antibody claimed in Claim 2 wherein the N-terminal peptide of GPC 3 is a peptide comprising amino acid residues 1-374 of GPC 3 or a peptide comprising amino acid residues 1-358 of GPC 3.

4. The antibody claimed in Claim 3 wherein the N-terminal peptide of GPC 3 is a peptide comprising amino acid residues 1-358 of GPC 3.

- 5. The antibody claimed in any one of Claims 1-4 which is a monoclonal antibody.
- 6. The antibody claimed in Claim 1 which is immobilized to an insoluble support.
- 7. The antibody claimed in Claim 1 which is labeled with a labeling material.
- 20 8. An antibody against a C-terminal peptide of GPC 3.
 - 9. The antibody claimed in Claim 8 wherein the C-terminal peptide of GPC 3 is a peptide comprising amino acid residues 359-580 of GPC 3 or a peptide comprising amino acid residues 375-580 of GPC 3.
- **10.** The antibody claimed in Claim 8 wherein the C-terminal peptide of GPC 3 is a peptide comprising amino acid residues 359-580 of GPC 3.
 - 11. The antibody claimed in any one of Claims 8-10 which is a monoclonal antibody.
- 30 12. The antibody claimed in any one of Claims 8-10 which is a chimera antibody.
 - 13. The antibody claimed in any one of Claims 8-10 which is a cytotoxic antibody.
 - 14. A cell disrupting agent comprising the antibody claimed in any one of Claims 7-13.
 - 15. The cell disrupting agent claimed in Claim 14 wherein the cell is a cancer cell.
 - 16. An anti-cancer agent comprising the antibody claimed in any one of Claims 8-13.
- 40 17. A method for inducing cytotoxicity comprising contacting a cell with the antibody claimed in any one of Claims 8-13.
 - 18. The method claimed in Claim 17 wherein the cell is a cancer cell.

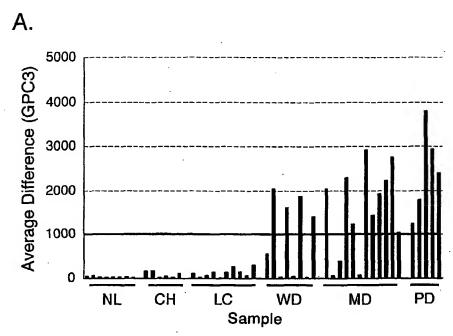
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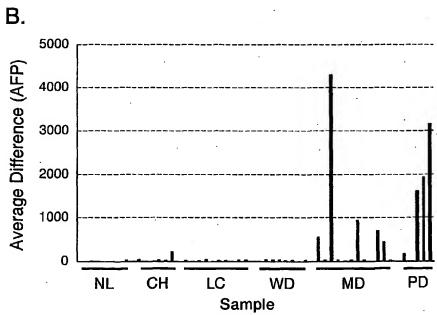
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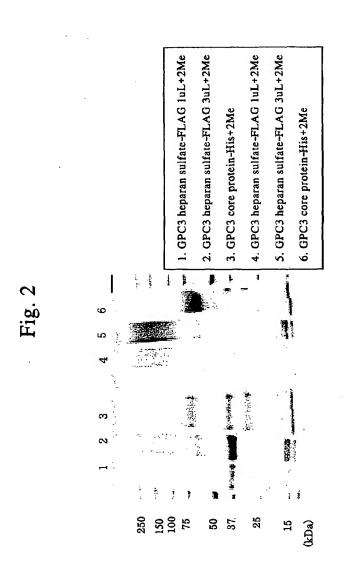
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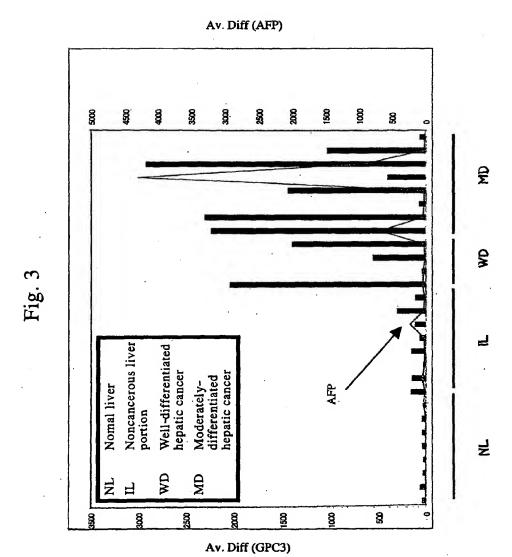
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Fig. 1









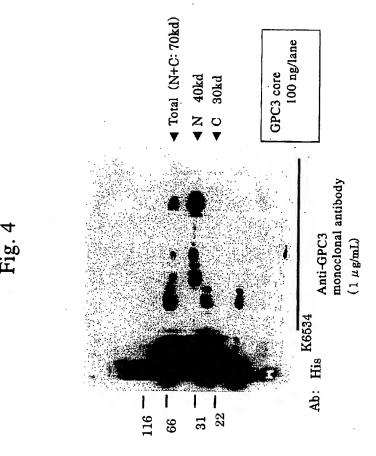


Fig. 5

OD measurement

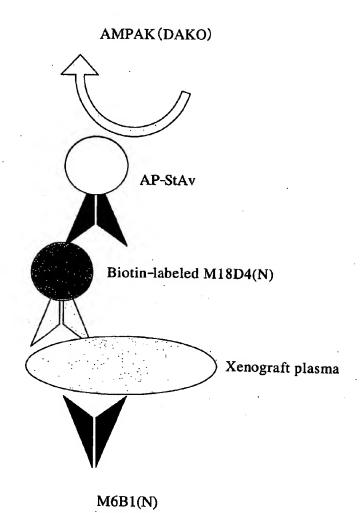


Fig. 6

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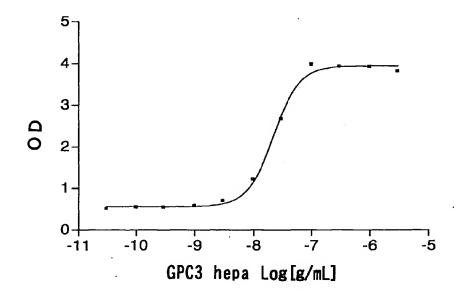
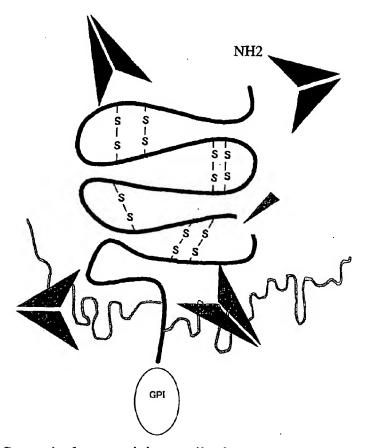


Fig. 7

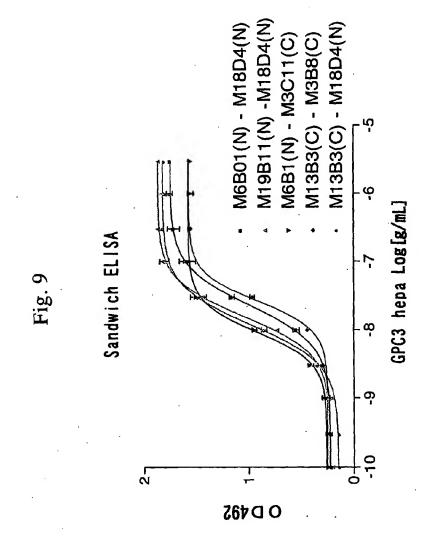
N-terminal-recognizing antibody

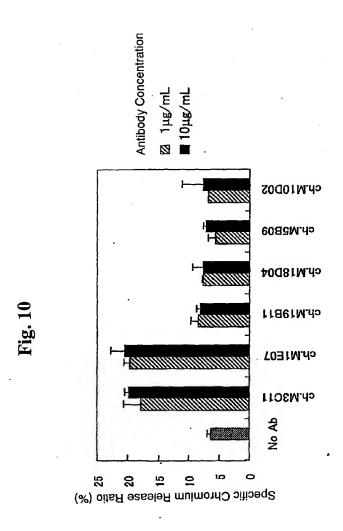


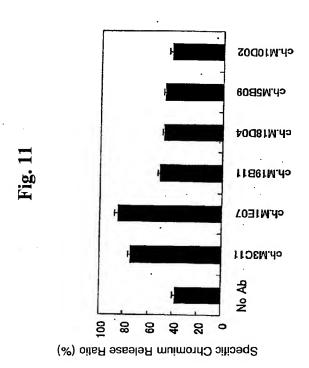
C-terminal-recognizing antibody

Fig. 8

	Form of soluble GPC3			
	N-terminus only	N+C	C-terminus only	
N-N ELISA	+	+		
N-C ELISA	*	+		
C-C ELISA		+	+	







EP 1 541 680 A1

International application No. INTERNATIONAL SEARCH REPORT PCT/JP03/11318 A. CLASSIFICATION OF SUBJECT MATTER Int.Cl7 C12N15/06, C07K16/18 According to International Patent Classification (IPC) or to both national classification and IPC B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) Int.Cl7 C12N15/00-15/90, C07K16/00-16/46 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) BIOSIS/MEDLINE/WPIDS(STN), JSTPlus(JOIS) C. DOCUMENTS CONSIDERED TO BE RELEVANT Category* Citation of document, with indication, where appropriate, of the relevant passages Relevant to claim No. CAPPURO M.I. et al., 'Overexpression of glypican-3 in human hepatocellular carcinomas determined by 1-13 immunohistochemistry using a monoclonal antibody', Proceeding of the American Association for Cancer Research Annual Meeting, March 2002, Vol. 43, page 219 1-16 P,X WO 03/000883 Al (Chugai Pharmaceutical Co., Ltd., Hiroyuki ABURAYA), 03 January, 2003 (03.01.03), (Family: none) P,X WO 03/010336 A2 (DEBUSCHEWITZ S., JOBST J., 1-13 KAISER S.), 06 February, 2003 (06.02.03), Page 21, Accession Nr.L47, 125.1 (Family: none) Further documents are listed in the continuation of Box C. See patent family annex. later document published after the international filing date or Special categories of cited documents: priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) step when the document is taken alone document of particular relevance; the claimed invention cannot be "Ľ" considered to involve an inventive step when the document is combined with one or more other such documents, such document referring to an oral disclosure, use, exhibition or other combination being obvious to a person skilled in the art document member of the same patent family document published prior to the international filing date but later than the priority date claimed Date of the actual completion of the international search Date of mailing of the international search report 04 November, 2003 (04.11.03) 23 October, 2003 (23.10.03) Name and mailing address of the ISA/ Authorized officer Japanese Patent Office Telephone No. Facsimile No.

Form PCT/ISA/210 (second sheet) (July 1998)

EP 1 541 680 A1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP03/11318

C (Continua	tion). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant p	Relevant to claim No.	
P,X	MIDORIKAWA, Y. et al., 'Glypican-3, overexp. in hepato-cellular carcinoma, modulates FGF. BMP-7 signaling', International Journal of 10 February, 2003 (10.02.03), Vol.103, No.4 pages 455 to 465	2 and Cancer,	1-13
P, X	SUNG Y.K. et al., 'Glypican-3 is overexpressed in human hepatocellular carcinoma', Cancer Science, March 2003, Vol.94, No.3, pages 259 to 262		1-13
P,X	CAPURRO M. et al, 'Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma', GASTROENTEROLOGY, July 2003, 125(1), 89-97		1–13
А	LAGE H. et al., 'Cloning and characterizati human cDNAs encoding a protein with high ho to rat intestical development protein OCI-5 Gene, 188(1997), 151-156	mology	1-16
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Form PCT/ISA/210 (continuation of second sheet) (July 1998)

EP 1 541 680 A1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP03/11318

Box I Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)
This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:
1. X Claims Nos.: 17, 18
because they relate to subject matter not required to be searched by this Authority, namely: Claims 17 and 18 involve methods for treatment of the human body by therapy and diagnostic methods and thus relate to a subject matter which this International Searching Authority is not required, under the provisions of Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search. 2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).
Box II Observations where unity of invention is lacking (Continuation of item 3 of first sheet)
This International Searching Authority found multiple inventions in this international application, as follows:
į,
As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Remark on Protest
No protest accompanied the payment of additional search fees.

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